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| | |
|---|---|
| ADP, CDP, GDP, IDP, UDP | 5′-Pyrophosphates of adenosine, cytidine, guanosine, inosine and uridine |
| AMP etc. | Adenosine 5′-phosphate etc. |
| ATP etc. | Adenosine 5′-triphosphate etc. |
| CoA | Coenzyme A |
| DNA | Deoxyribonucleic acid |
| DPN | Diphosphopyridine nucleotide |
| EDTA | Ethylenediaminetetra-acetate |
| FMN | Flavin mononucleotide |
| FAD | Flavin–adenine dinucleotide |
| GSH, GSSG | Glutathione, reduced and oxidized |
| NAD | Nicotinamide–adenine dinucleotide |
| NADP | Nicotinamide–adenine dinucleotide phosphate |
| NMN | Nicotinamide mononucleotide |
| RNA | Ribonucleic acid |
| TPN | Triphosphopyridine nucleotide |
| tris | 2-Amino-2-hydroxymethylpropane-1:3-diol |

*Abbreviations for amino acids and carbohydrates.* Those marked * and † in *Suggestions to Authors* are to be used only for representation of polymers or sequences and in Tables and Figures.

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*Biochem. J.* (1961) **81**, 225



# Protein Turnover and the Formation of Protein Inclusions during Sporulation of *Bacillus thuringiensis*

By R. E. MONRO*

*Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge*

(*Received* 10 *February* 1961)

During sporulation of *Bacillus thuringiensis*, a spore and a protein parasporal inclusion are formed within each cell (Hannay & Fitz-James, 1955; Hannay, 1956). The parasporal inclusions are of a regular, dipyramidal form and are referred to as 'crystals'. Similar 'crystals' are formed by a number of related strains of *Bacillus*, all of which are pathogenic to insect larvae. The crystals are composed of protein, and contain no nucleic acid or carbohydrate. The crystals are insoluble in water and do not disperse until the pH is raised to a value above 11·8. After such dispersal, the crystal protein remains in solution over a range of pH values from 6 upwards.

It has been shown (Monro, 1961) that normal sporulation with concomitant formation of protein crystals can take place during incubation under suitable conditions in a nutrient-free medium. It has further been shown that crystal protein antigens are absent from vegetative cells and arise over the same period as the crystals during sporulation. It is thus clear that the crystal protein must be derived from material already present in the cells before sporulation but that such precursor material is antigenically unrelated. In the present paper, chemical analysis and tracer techniques have been employed to investigate whether crystals are formed through modification of antigenically unrelated protein or polypeptide components, or whether proteins break down during sporulation, supplying amino acids for synthesis of the crystal protein. With the aid of a serological method, which has been developed for the rapid, reproducible separation of the crystal protein, it has been found that the crystal protein is synthesized from amino acids during sporulation, and that a process of protein turnover takes place. These results are in agreement with related observations of Young & Fitz-James (1959), who have shown by somewhat similar techniques that the crystal protein of a related strain is synthesized during sporulation.

Further studies show that a general process of protein turnover occurs during sporulation under the conditions employed and that, as well as the crystal protein, a variety of other new proteins are also synthesized. The subject of protein turnover has been reviewed by Mandelstam (1960).

## METHODS

The strain of *B. thuringiensis* used throughout the present study, as well as the cultural and sporulation conditions, are described elsewhere (Monro, 1961). Cells were grown in a liquid casein-digest medium to the early stationary phase of growth. The washed cells were then resuspended in a nitrogen-free, buffered salts medium and incubated with aeration. Synchronous sporulation took place in over 75% of the cells within 10 hr.

Light-microscopy, ultrasonic treatment and methods used for the preparation of parasporal inclusions are described elsewhere (Monro, 1961).

*Dry weight.* Samples of culture were centrifuged in tared tubes. The cells were washed once with water and the sediments dried for 8 hr. at 105°. After cooling over $H_2SO_4$ the tubes with samples were weighed.

*Free amino acid pool.* Samples of culture were rapidly filtered on Oxoid membrane filters and washed twice with M-NaCl. The filter was then removed to a centrifuge tube containing cold 0·2 N-$HClO_4$ and the cells were dispersed. After 30 min. at 0° the tubes were centrifuged and the ninhydrin-reacting material was estimated by the method of Yemm & Cocking (1955). Results are expressed as $\mu$moles of free amino acids by reference to an alanine standard. Chromatography of extracts showed that over 90% of the ninhydrin-positive material consisted of free amino acids.

*Nucleic acid estimation.* DNA was estimated by the method of Burton (1956). RNA was estimated from the extinction at 260 m$\mu$ of $HClO_4$ extracts of cells (Ogur & Rosan, 1950). Hot and cold extracts were combined since RNA was only partially extracted in the cold. The absorption spectrum of such extracts over the range 215–300 m$\mu$ was measured and shown to be typical of nucleic acids. Absorption due to DNA was allowed for in calculating the RNA content. The extinction coefficient of a 0·1% solution of RNA, treated with $HClO_4$ as above, has been assumed to be 28 (Gale & Folkes, 1953).

*Alkali fractionation.* This has been carried out for spores and crystals by a modification of the method used by Fitz-James, Toumanoff & Young (1958). Sporulated cultures were stored at 5° for 1–2 days, under which conditions vegetative-cell walls and membranes disperse, releasing the spores and crystals. Remaining debris was removed by repeated centrifuging, under conditions adjusted to sediment the spores and crystals but to leave debris in the supernatant. The initial centrifuging and wash were per-

* Present address: Institute for Molecular Biology, Cambridge.

formed at 5° to avoid germination, which is stimulated by the presence of the accumulated breakdown products (Halvorson, 1958). Crystals were removed quantitatively from such spore–crystal mixtures by extraction with alkali. Spores and crystals were suspended in 0·1 N-NaOH, washed once with 0·1 N-NaOH and then twice with water. Viable spores were left behind and there was no trace of the crystals remaining in the sediment. The supernatants were combined and the crystal protein was precipitated by adjustment of the pH to a value of 5·0.

*Peptide 'map'.* Hydrolysates obtained by tryptic digestion of crystals were examined by high-voltage paper electrophoresis under toluene. Crystals were suspended in citrate–phosphate buffer, pH 3 (McIlvaine, 1921), incubated for 10 min. at 100° and then washed with water. The denatured crystals were resuspended at a concentration of 5 mg. dry wt./ml. in 2% (w/v) $NH_4HCO_3$ containing trypsin (50 $\mu$g./ml.). The suspension was incubated at 38° for 100 min. The freeze-dried digest was subjected to electrophoresis on Whatman no. 1 paper, with a buffer that consisted of pyridine (200 ml.), acetic acid (8 ml.) and water to 2 l. (pH 6·5). It was run for 120 min. at 40 v/cm.

*Serological separation of crystal protein.* Disrupted cells were extracted with alkali, the insoluble residue (spores and some cell walls) was taken as the spore fraction, and the crystal fraction was obtained by treatment of the supernatant with crystal antiserum. The procedure adopted was as follows: the sample of culture (0·25–0·5 ml.) was exposed to ultrasonic vibration for 1 min. in a volume of about 1 ml. (rods disrupted but crystals and mature spores left intact) and then centrifuged in a 3·5 ml. tube. The supernatant was discarded and the sediment suspended in 0·1 ml. of 0·1 N-NaOH and incubated for 2 hr. at about 20°. The pH was then brought to 7·5 by addition of 0·2 ml. of phosphate buffer, and the suspension was centrifuged. The sediment was taken as the 'spore fraction'. Serum [0·5 ml.; third course anti-crystal serum (Monro, 1961) diluted tenfold with 0·9% NaCl] was added to the supernatant, and the mixture incubated for 1 hr. at 37° with occasional gentle agitation; the suspension was then left overnight at 5°. After centrifuging, the supernatant was discarded, and the sediment taken as the 'crystal fraction'. For estimation of radioactivity spore and crystal fractions were suspended in N-$NH_3$ solution and dried on planchets.

By use of a purified preparation of $^{14}$C-labelled crystals to which unlabelled, sporulated culture was added in variable amount, it was shown that all of the crystal protein was extracted from the spore fraction and over 80% recovered in the crystal fraction. The specificity of the method was also investigated. A culture was labelled with [$^{14}$C]phenylalanine and samples were taken before and after sporulation. Unlabelled, sporulated culture was added as carrier and the crystal fraction isolated. The amount of radioactivity found in the crystal fraction from the vegetative cells was less than 20% of that from the sporulated cells. It is probable that the small amount of protein recovered in the crystal fraction from vegetative cells was due mainly to the presence of small amounts of vegetative-cell antibodies in the serum (Monro, 1961).

*Radioactivity of protein.* The protein fraction was isolated by a modification of the method of Schneider (1945). The sample was centrifuged and the sediment suspended in 5% trichloroacetic acid containing about 2 mg. of DL-phenylalanine/ml. After incubation at 90° for 20 min., the suspension was centrifuged, and the sediment washed with acidified ethanol and with ether (radioactivity in ethanol and ether supernatants was negligible). The sediment was allowed to dry and was then dispersed in 250 $\mu$l. of N-$NH_3$ solution and left overnight at 5°. A sample (200 $\mu$l.) was dried on a planchet.

## RESULTS

*Chemical analysis.* Estimates of the intracellular content of free amino acids during growth and sporulation are shown in Table 1. The content of free amino acids in sporulating cells was less than that in growing cells. There was a significant increase in intracellular amino acids over the initial stages of sporulation, and during the later stages there was a fall.

An estimate was made of the dry weights of crystals and spores formed during sporulation. A culture was allowed to sporulate. The spores and crystals were freed from vegetative-cell remnants and then separated into spore and crystal fractions by alkali treatment (see Methods). Duplicate dry-weight determinations were made (*a*) on cells immediately after transfer, (*b*) on the spore–crystal fraction, (*c*) on the spore fraction, and (*d*) on the precipitated crystal protein. All fractions were washed once with water before drying. Electron-microsopic examination showed that the fractions were nearly pure.

Table 2 shows the results of the analyses. The crystal fraction, estimated from the precipitated protein, amounted to only about 73% of the estimates obtained by difference. The actual amount of crystal protein presumably lies between the extremes of these two estimates. Table 2 also shows the results expressed as percentage of the initial dry weight of cells and a correction is applied to

Table 1. *Intracellular content of free amino acids*

For analytical procedure, see Methods.

| Culture | Time after transfer to sporulation medium (hr.) | Intracellular amino acids* ($\mu$moles/g. dry wt. of cells) |
|---|---|---|
| Exponential | — | 340 |
| Exponential | — | 404 |
| Sporulating | 0 | 144 |
| | 0 | 141 |
| | 2 | 175 |
| | 2 | 174 |
| | 4 | 164 |
| | 6 | 168 |
| | 6 | 167 |
| | 8 | 142 |
| | 8 | 131 |

* For sporulating cells, amino acid content is referred to as dry wt. of cells in culture at time 0.

Table 2. *Dry weights of cells, spores and crystals*

The procedure followed is described under Methods.

| | Dry weight (mg./ml. of culture) | Percentage of initial dry weight | |
|---|---|---|---|
| | | Actual | Corrected* |
| (*a*) Cells at time of transfer | 1·73<br>1·75 | 100 | 100 |
| (*b*) Spore–crystal fraction | 0·42<br>0·42 | 24·1 | 32·0 |
| (*c*) Spore fraction | 0·198 | 11·4 | 15·2 |
| (*d*) Crystal fraction by difference (*b* − *c*) | 0·22 | 12·6 | 16·8 |
| (*e*) Precipitated crystal protein fraction | 0·149<br>0·171 | 9·2 | 12·3 |

* Cell counts on the same culture showed that only about 75% of the cells gave rise to spores and crystals. Hence a correction factor of 100/75 is employed.

Table 3. *Deoxyribo- and ribo-nucleic acid content*

Estimates are expressed as percentages, referred to the dry wt. of cells at the time of transfer to the sporulation medium.

| Time after transfer to sporulation medium (hr.) | DNA (%) | RNA (%) |
|---|---|---|
| 0 | 1·18 | 6·06 |
| | 1·14 | 6·03 |
| 2 | 1·10 | 4·87 |
| | 1·08 | 4·88 |
| 4 | 1·06 | 4·25 |
| | 1·06 | 4·33 |
| 6 | 1·04 | 4·08 |
| | 1·04 | 4·03 |
| 8 | 1·02 | 3·84 |
| | 1·00 | 3·90 |

Table 4. *Incorporation of added amino acids during growth*

A sample (0·5 ml.) of culture (early exponential phase) was transferred to a 25 ml. specimen tube containing the labelled amino acid (5–15 c/mole); subsequent growth and sporulation were as normal. Radioactivity in spore and crystal fractions was measured after separation by the serological method. The amount of each amino acid added was 80 000 counts/min.

| Amino acid | Counts/min. recovered in Crystal fraction | Spore fraction |
|---|---|---|
| Aspartic acid | 240 | 1470 |
| Glutamic acid | 370 | 2950 |
| Proline | 140 | 245 |
| Leucine | 110 | 215 |
| Isoleucine | 70 | 140 |
| Phenylalanine | 150 | 325 |
| Tyrosine | 80 | 80 |
| Serine | 160 | 925 |
| Threonine | 870 | 2085 |

allow for cells that did not sporulate. Estimates are minimal in view of the unknown loss (less than 25%) of spores and crystals during differential centrifuging. The spore fraction is subject to a further underestimate as a result of germination and autolysis which occurred in an undetermined percentage of spores during storage at 5° and after ultrasonic treatment. Bearing these errors in mind, it is clear that the dry weight of the average spore and crystal amount to over 15% and 12·5–17% respectively of the dry weight of the cell from which they are formed. It can also be calculated that less than 15% of the crystal protein could arise from free amino acids present in cells at the time of transfer to the sporulation medium.

Table 3 shows that the DNA content of cultures remains nearly constant over the course of sporulation. In contrast, RNA content falls by about 40%.

*Tracer studies*

$^{14}$C-Labelled amino acids, assimilated by cells during vegetative growth, are found in the crystals that are formed during subsequent sporulation (Table 4). Such incorporation is to be expected, since crystals are derived entirely from material present in vegetative cells. However, amino acids added during sporulation are also incorporated into the crystal protein (Table 5). It therefore follows that at least part of the crystal protein is synthesized *de novo* from amino acids during sporulation.

*Peptide 'map'*. Models can be envisaged in which one part of the protein crystal is synthesized during vegetative growth and a distinct part during sporulation: namely (i) a protein present in vegetative cells is modified by attachment of additional amino acids to it during sporulation, or (ii) the crystals are composed of two proteins, one of which is synthesized during growth and the other during sporulation. Such possibilities have

Table 5. *Incorporation of amino acids added during sporulation*

Sporulating culture (0·25 ml.) was pipetted into a 25 ml. specimen tube containing the labelled amino acid (5–15c/mole). After a period of incubation, excess of non-radioactive amino acid was added (giving a final concentration of about 2 mg./ml.) and incubation continued until sporulation was complete. Spore and crystal fractions were separated by the serological method and counted.

| Amino acid | Period of exposure (time after transfer) (hr.) | Counts/min. added | Counts/min. recovered in: Crystal fraction | Counts/min. recovered in: Spore fraction |
|---|---|---|---|---|
| Glutamic acid | 2–10 | 33 000 | 738 | 1380 |
| Proline | 2–4 | 30 000 | 834 | 1184 |
| Phenylalanine | 2–2·5 | 15 000 | 493 | 360 |

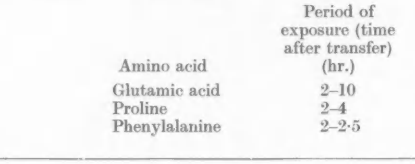


Fig. 1. Peptide 'maps' of labelled crystals. Crystal preparations: X, Cells grown in the presence of [$^{14}$C]glutamate (10 $\mu$c added to growth medium) and then sporulated as usual; Y, unlabelled culture sporulated in the presence of [$^{14}$C]glutamate (5 $\mu$c added 3·5 hr. after transfer). Crystals were separated from both cultures by the method of isopycnic centrifuging. Peptide 'maps' were prepared as described under Methods. Tryptic digests of the crystal preparations were put on the paper in two adjacent 1·2 cm. strips (at points indicated by arrows). After electrophoresis, a radioautograph was taken. In taking the photograph of the radioautograph (shown here), it was necessary to expose the bands from preparation X for a longer period than those from Y. Hence the slight discontinuity in certain regions of the photograph and the differences in background density.

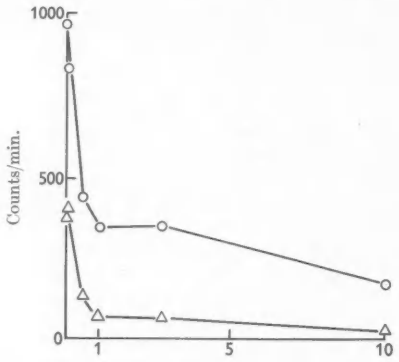


Fig. 2. Radioactivity of spore fractions (○) and crystal fractions (△) after sporulation of cells prelabelled with [$^{14}$C]phenylalanine: effect of addition of [$^{12}$C]phenylalanine to the sporulation medium. Cells were prelabelled with [$^{14}$C]phenylalanine by the procedure described in the text. Samples (0·25 ml.) were then sporulated in the presence of various concentrations of [$^{12}$C]phenylalanine. After sporulation was complete, excess of [$^{12}$C]phenylalanine was added and the radioactivity of the spore and crystal fractions estimated by the serological method.

been investigated by studying incorporation into specific regions of the crystal protein molecule(s). Crystals were prepared by the method of isopycnic centrifuging (Monro, 1961) from cells labelled by addition of [$^{14}$C]glutamate (*a*) during vegetative growth and (*b*) during sporulation. Tryptic digests of the crystals were subjected to electrophoresis on paper and the resulting distribution of tracer was examined by radioautography. Fig. 1 shows that the same peptides are labelled whether [$^{14}$C]-glutamate is added during growth or sporulation. It can be concluded that incorporation of amino acids added during sporulation results from a general synthesis of crystal protein and not the synthesis of a specific part of the crystal protein. It is not clear from these results what percentage of the crystal protein is synthesized during growth and what percentage during sporulation.

*Labelling of vegetative cells.* In subsequent experiments cells were labelled with [$^{14}$C]phenylalanine. Unless otherwise stated, cells from exponential phase culture were resuspended in a synthetic medium containing 17 amino acids (lacking phenylalanine). After incubation for 10 min., [$^{14}$C]phenylalanine (giving 24 $\mu$g./ml. and specific activity as required) was added. After further incubation for 1 hr. cells were resuspended in growth medium. Subsequent growth and sporulation were normal. Incubation after retransfer to the growth medium was for approximately 4 hr. and there was a three- to four-fold increase in dry weight. Chromatography and radioautography of hydrolysates of cells labelled

in this way showed that approximately 80% of the label was present as phenylalanine, 10% as tyrosine, and 10% as glutamic and other amino acids.

*Transfer experiment.* It is clear from the 'peptide map' experiment described above, that there is at least some *de novo* synthesis of crystal protein during sporulation. The extent of such synthesis has been investigated by studying the incorporation of tracer into crystals during sporulation of prelabelled cells. It should be possible to prevent the transfer of a given labelled amino acid from old to newly synthesized protein by 'swamping' with excess of the corresponding unlabelled amino acid. The percentage fall in radioactivity of the crystal under such conditions would then give a measure of the percentage of the crystal protein that is newly synthesized.

Vegetative cells have been labelled with [$^{14}$C]-phenylalanine and then sporulated in the presence or in the absence of unlabelled phenylalanine. Fig. 2 shows that the crystals formed in the presence of unlabelled phenylalanine were of greatly reduced radioactivity. Thus the counts/min. were reduced by 85–90% when DL-phenylalanine was added at concentrations of 1–10 mg./ml. The corresponding counts in the spore fraction were lowered by 70–80%. Microscopic examination revealed no differences between sporulation in the presence and in the absence of phenylalanine. The possibility is thus eliminated that the fall in radioactivity of the crystal fraction was due to adverse effects of phenylalanine upon sporulation. It can be concluded that at least 80% of the crystal protein is synthesized *de novo* during sporulation from amino acids which arise through the breakdown of other proteins.

*General turnover of protein during sporulation.* This was followed by measuring the radioactivity of the total protein and by taking samples at intervals rather than just at the end of sporulation. By this means estimates can be made for the breakdown of protein as well as for the synthesis of new protein (Fig. 3). Cells were labelled with [$^{14}$C]phenylalanine, and sporulated in the presence or in the absence of unlabelled amino acids (D- and L-phenylalanine, L-tyrosine and L-glutamate at concentrations of 10 mM each). Samples were

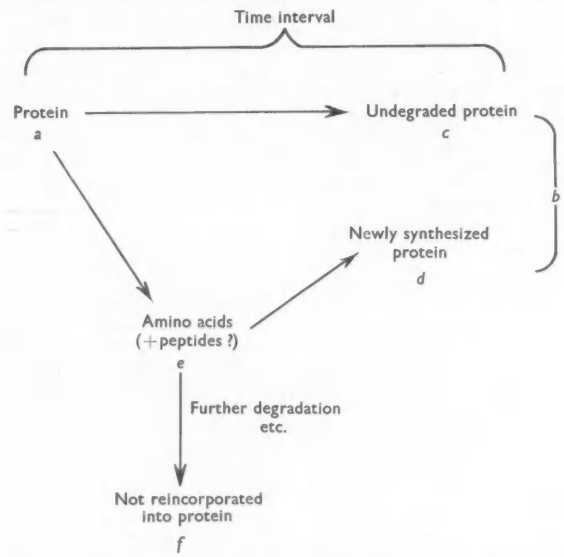


Fig. 3. Scheme to illustrate the method used for estimation of protein turnover. The protein is initially labelled with a [$^{14}$C]amino acid 'A', and the cells are incubated in the presence or in the absence of excess of unlabelled 'A'. In the former case any labelled 'A' is diluted by the added 'A' and reincorporation of the label into new protein thus prevented. If, then, radioactivity is taken as a measure of the amount of protein containing labelled 'A', direct estimates are made for *a* (initial protein), *b* (labelled protein after incubation in the absence of unlabelled 'A'), and *c* (labelled protein after sporulation in the presence of 'A'). Further: newly synthesized protein = $b - c$; protein broken down = $a - c$; amino acid not reincorporated = $a - b$. Assumptions involved in this scheme are considered in the Discussion.

taken at intervals and the radioactivity of the protein was estimated (see Methods).

Both microscopic examination and analysis for protein (Lowry *et al.* 1951) revealed no differences between cells that sporulated in the absence and in the presence of added amino acids. It is therefore improbable that the presence of the added amino acids had an appreciable effect on the rates of breakdown or resynthesis of proteins in the sporulating cells.

The course of turnover during sporulation is shown in Fig. 4. In the presence of added amino acids there was a continued, rapid fall in the counts/min. in the protein over the first 8 hr. of incubation. There was much less fall with the unsupplemented medium. In Fig. 5 the results are plotted in a different way, showing the course of breakdown and resynthesis. The rates of breakdown and resynthesis were both about 10 %/hr. over the first 2 hr. After 2 hr. these processes continued at a diminished rate. The rate of synthesis fell more rapidly than the rate of breakdown, the difference in rates being accounted for by breakdown products' not being reincorporated into the protein fraction. After 8 hr. little further synthesis took place, whereas breakdown continued at a slow rate (about 3·3 %/hr.) between 8 and 13 hr. Estimates for breakdown and resynthesis at 13 hr. amount to 67 and 52 % respectively. As indicated, estimates for turnover have varied somewhat from one experiment to another. In particular, cultures occasionally lysed to a greater extent than normal and the amount of resynthesis was then correspondingly lower. These results are considered further in the Discussion.

In order to gain information about the nature of the proteins that break down during sporulation, estimates of breakdown have been made on cells labelled at different phases of growth. [$^{14}$C]-Phenylalanine was added directly to the growth medium in order to eliminate the possibility of abnormal distribution of protein synthesis during incubation in the synthetic medium (used for incorporation in other experiments). The results (Table 6) show that proteins synthesized during the

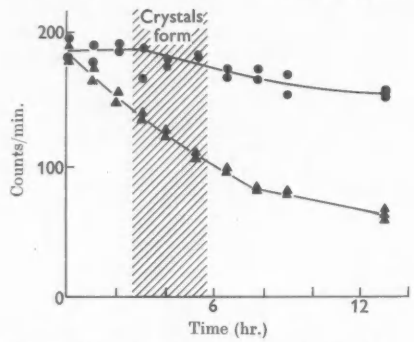


Fig. 4. Turnover of protein during sporulation. Cells were labelled with [$^{14}$C]phenylalanine and then sporulated in the presence or in the absence of unlabelled amino acids (D- and L-phenylalanine, L-tyrosine and L-glutamate at concentrations of 10 mM each). Samples were taken at intervals for the estimation of radioactivity in the protein fraction. Each point represents a single determination. ●, No amino acids added to sporulation medium; ▲, amino acids added. Cell counts at 13 hr. showed that about 80 % of the cells had sporulated, whereas the others appeared to be cell walls without contents.

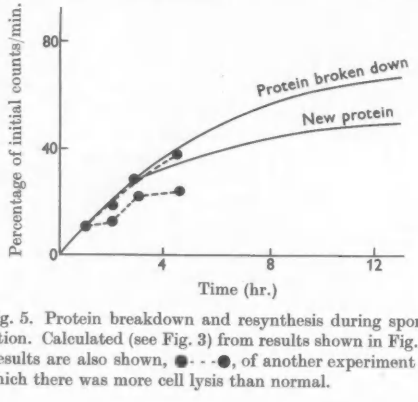


Fig. 5. Protein breakdown and resynthesis during sporulation. Calculated (see Fig. 3) from results shown in Fig. 4. Results are also shown, ●- - -●, of another experiment in which there was more cell lysis than normal.

Table 6. *Breakdown of protein: effect of labelling at different phases of growth*

Samples (2·5 ml.) of culture (turbidity 0·02 arbitrary unit) were pipetted into each of three flasks and 0·5 $\mu$c of [$^{14}$C]phenylalanine was added to the first flask. When the turbidity reached 0·20, the first and second cultures were centrifuged, the supernatants exchanged and the cells resuspended. The second and third cultures were 'exchanged' in a similar manner when the turbidity reached 0·40. Cultures were transferred at the usual stage of growth to the sporulation medium to which phenylalanine, tyrosine and glutamate were added, as in the previous experiment. Samples (0·5 ml.) were taken at 0 and 5 hr. after transfer for estimation of radioactivity in the protein.

| Turbidity at period of exposure to tracer | Counts/min. 0 hr.* | Counts/min. 5 hr.* | Undegraded protein (% of initial counts/min. remaining in protein) at 5 hr.* |
|---|---|---|---|
| 0·02–0·20 | 105 | 59 | 55 |
| | 117 | 63 | |
| 0·20–0·40 | 73 | 52 | 63 |
| | 74·5 | 41 | |
| 0·40–0·65 | 140 | 108 | 78·5 |
| | 129 | 104 | |

* Time after transfer.

log phase of growth, as well as those synthesized during the transition and early stationary phases, break down during sporulation. There is some indication that proteins synthesized in the early stationary phase break down to a lesser extent than those synthesized in the log phase, but this difference is probably not significant.

## DISCUSSION

The chemical analyses show that less than 15% of the synthesis of crystal protein could be accounted for by the free amino acids present in cells before sporulation. Serological work reported elsewhere (Monro, 1961) shows that the crystals are not formed by simple crystallization of protein present in vegetative cells. Further, the 'peptide map' experiment shows that formation of the crystals does not take place through synthesis of one part of the protein crystal during vegetative growth and a distinct part during sporulation. The transfer experiment gave a value of 80% for the amount of crystal protein synthesized *de novo* from amino acids during sporulation. This figure is a minimal estimate in view of the following possible sources of error: (*a*) incomplete equilibration between intracellular and added amino acids; (*b*) approximately 20% of the phenylalanine is converted into other amino acids during incorporation. The transfer of label in the form of these other amino acids to newly forming protein would not necessarily be 'swamped' by the addition of unlabelled phenylalanine since rapid interconversion would be necessary for such 'swamping' to be efficient; (*c*) the precipitation of small amounts of vegetative-cell proteins in the crystal fraction; this contamination may have been as much as 20% of the fraction (see Methods) and could by itself account for the residual counts obtained in the crystal fraction of cells that sporulated in the presence of phenylalanine.

It can be concluded that most, if not all, of the crystal protein is synthesized from amino acids during sporulation. In conjunction with the chemical analyses these results lead to the view that amino acids used for synthesis of the crystal protein are supplied through a breakdown of protein. Crystals, then, can be considered to be formed by a process of 'protein turnover'.

Errors in the time-course experiment could have arisen from the method of labelling used. Thus the label was incorporated only at a particular phase of growth and under abnormal conditions. Such conditions could have led to labelling of only particular protein fractions of the cell. Similar amounts of breakdown to those estimated in the above experiment were, however, estimated for cells labelled at a comparable phase of growth by addition of [$^{14}$C]phenylalanine to the normal growth medium, and it can therefore be concluded that error did not result from abnormality of labelling conditions. There is a possibility, however, that proteins synthesized during the exponential phase of growth are more labile during sporulation than those synthesized during the transition phase. From the figures in Table 6 it is estimated that this effect could lead to as much as 15% overestimation of turnover.

There is a possibility that proteins synthesized during sporulation are subject to breakdown as are the other proteins in the cell. If this is the case, then the actual amounts of turnover will have been underestimated but the estimates will apply to the net amounts of new and old proteins, referred to the protein content of cells at the time of transfer.

The [$^{14}$C]phenylalanine content of proteins has been taken as an estimate of the amount of protein containing the label. If there are different percentages of phenylalanine in the initial protein, in the protein that breaks down and in the protein that is resynthesized, then resulting estimates for breakdown and resynthesis would be correspondingly biased.

It is considered by some workers (Young & Fitz-James, 1959) that normal sporulation cannot take place in nutrient-free media. It is held that sporulation in such systems only takes place through the supply of nutrients by lysis of a portion of the cells. However, evidence has been presented elsewhere (Monro, 1961) that normal sporulation took place in the system used in the present studies. All of the cells could be accounted for by microscopic cell counts, and sporulation took place in over 75% of the cells. Negligible cell lysis took place during the early stages of sporulation in many experiments. It might be argued that the conservation of cell numbers could be explained on a basis of turnover on a cellular level, but this possibility is eliminated by the high degree of synchrony observed in sporulating cultures. Further evidence for normal sporulation is provided by the DNA estimations presented in this paper, which are closely similar to those obtained by Young & Fitz-James (1959) for cells sporulating in a nutrient medium.

In the time-course experiment on protein turnover described in Fig. 5, negligible cell lysis was observed during the first 2 hr. It follows that turnover estimations for this period are not confused by lysis. After 2 hr., however, internal lysis took place in a small percentage of the cells, and this lysis amounted to about 20% at 10 hr. Lysis will lead to underestimation of the amount of resynthesis and overestimation of the amount of breakdown that takes place in healthy cells.

Further, the possibility arises that turnover is not entirely an intracellular process, but that synthesis of new protein takes place partly through utilization by sporulating cells of breakdown products from cells which lyse. However, the amount of new protein synthesized could not be accounted for by the amount of cell lysis observed, and it seems probable that turnover taking place during the late as well as early stages of sporulation is an intracellular process. Detailed calculations (Monro, 1959) show that all of the synthesis during sporulation could be accounted for by intracellular turnover, but breakdown products of lysed cells could also have contributed to making up to 50 % of the new protein. It is also estimated that during sporulation of a cell, new protein is synthesized, which amounts to about 50 % of the protein present in the cell at the time of transfer.

The studies on protein turnover show that crystal formation is part of a general process occurring during sporulation, in which there is a radical transformation of the proteins of the cell. Estimates for the initial rate of turnover (about 10 %/hr.) are of the same order as those reported by Urbá (1959) for *Bacillus cereus*, incubated in a nutrient-free medium (about 7 %/hr.). Estimates for the extent of turnover during the whole of sporulation are about 50 % and, if correct, indicate a higher degree of protein turnover than in any system previously reported.

Foster & Perry (1954) have shown that protein degradation and resynthesis occur during endotrophic sporulation of *Bacillus mycoides* but this work is open to some criticism (Young & Fitz-James, 1959). It is probable that bacilli, capable of sporulation, are particularly well equipped for turnover in view of the radical intracellular changes during sporulation, which often takes place under conditions of poor nutrition. An association of well-developed machinery for turnover with the capacity to sporulate would fit in with the higher rates of protein turnover found for *Bacillus cereus* (Urbá, 1959) than for *Escherichia coli* (Mandelstam, 1958). It is possible that, concomitant with the turnover which Urbá observed in resting cell suspensions, the cells underwent the initial stages of sporulation.

The extent of protein breakdown which occurs during sporulation shows that much of the protein of the cell must be susceptible to breakdown rather than just a particular fraction. Further, breakdown is not confined to proteins synthesized during a particular phase of growth. It is probable that the breakdown of RNA during sporulation is even greater than that indicated by the analyses in view of the report by Fitz-James *et al.* (1958) that there is a turnover of RNA during sporulation of *Bacillus cereus* var. *alesti*. The RNA breakdown suggests a general disintegration of ribonucleoprotein particles and there could be a concomitant breakdown of the protein of these particles.

## SUMMARY

1. Studies have centred around the formation of protein, crystalline, parasporal inclusions and general protein metabolism in cells of *Bacillus thuringiensis* during sporulation in a nutrient-free medium. Analysis of the metabolic pool shows that insufficient free amino acids to account for synthesis of all the crystal protein are present at the time of transfer to the nutrient-free medium. $^{14}$C-Labelled amino acids, added either during growth or sporulation, are incorporated into the crystals. [$^{14}$C]Glutamic acid, whether it is added during growth or sporulation, is incorporated into the same positions in the polypeptide chains of the crystal protein. When cells are prelabelled with [$^{14}$C]phenylalanine, the addition of unlabelled phenylalanine to the sporulation medium prevents incorporation of label into the crystals. It is concluded that the crystal protein is synthesized from amino acids during sporulation and that, under the conditions employed, these amino acids are derived largely from the breakdown of vegetative-cell proteins. Further studies show that there is a high percentage of intracellular protein turnover during sporulation, and that other proteins, in addition to the crystal protein, are synthesized.

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# The Isolation and Characterization of *trans*-Octadec-16-enoic Acid from Butterfat

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*trans*-Octadec-16-enoic acid was first reported as a fatty acid constituent of butterfat by Backderf (1956) and Backderf & Brown (1958), who deduced its presence from infrared-spectrophotometric analyses supported by the conclusive identification of thapsic acid (hexadecanedioic acid) as the main dicarboxylic acid product from the oxidation of appropriate *trans* $C_{18}$ mono-unsaturated fractions with potassium permanganate and acetic acid. They did not, however, actually isolate *trans*-octadec-16-enoic acid. Formerly its presence in a natural fat had not been recorded although experimental evidence indicated that *trans* isomers of *n*-octadecenoic acid including 'vaccenic' acid were contained in cow milk fat (Bertram, 1928; Gupta, Hilditch, Paul & Shrivastava, 1950; Cornwell, Backderf, Wilson & Brown, 1953; Smith, Freeman & Jack, 1954; Scott, Herb, Magidman & Riemenschneider, 1959; Patton, McCarthy, Evans & Lynn, 1960), in sheep milk fat (Gupta *et al.* 1950), in ox body fat (Bertram, 1928; Rao & Daubert, 1948; Gupta *et al.* 1950; Swern, Knight & Eddy, 1952), and in sheep body fat (Bertram, 1928; Gupta *et al.* 1950).

The isolation and characterization of *trans*-octadec-16-enoic acid is reported in the present paper, which results from an investigation of the trace and minor fatty acid constituents of butterfat. The techniques employed included fractional distillation *in vacuo*, low-temperature crystallization, and purification by elution through a chromatographic column of activated silicic acid. Both the characterization of *trans*-octadec-16-enoic acid and the identification of thapsic acid resulting from its potassium permanganate oxidation have been supported by gas–liquid-chromatographic analyses.

## EXPERIMENTAL AND RESULTS

The butterfat sample used in this investigation was prepared from fresh summer butter supplied by the Awahuri Dairy Co., Palmerston North, New Zealand. From this sample *cis*-heptadec-9-enoic acid was recently isolated (Hansen, Shorland & Cooke, 1960).

The gas–liquid chromatogram used in this work was constructed in the Fats Research Laboratory and incorporates the $^{90}Sr$ ionization detector first described by Lovelock, James & Piper (1959). The glass column employed was 8 ft. long and $\frac{1}{4}$ in. in diameter and was packed with Celite (Chromosorb; Johns-Manville, U.S.A.) impregnated with 20% (w/w) polydiethylene glycol adipate. A second column impregnated with 5% (w/w) Apiezon L was used for the characterization of the *trans* acid isolated. The carrier gas was argon and the operating temperature 207°. All $R_F$ values reported in this paper were calculated relative to methyl stearate (methyl *n*-octadecanoate, $R_F$ 1·00) and peak areas represented the moles % of the components present.

X-ray measurements were made with a Phillips Geiger X-ray spectrometer with manganese-filtered iron K$\alpha$ radiation. Samples were melted on a glass slide and cooled.

Combustion and oxygen analyses were determined by Dr A. D. Campbell, Chemistry Department, University of Otago, Dunedin, New Zealand.

Melting points are uncorrected and were determined in closed capillaries for temperatures below 100°, whereas for temperatures above 100° determinations were made with the Hoover Uni-melt with capillaries open at one end.

Hydrolysis of 3502·5 g. of butterfat by the conventional method of Hilditch (1956), followed by steam-distillation, yielded 3099·0 g. of steam-non-volatile acids, which were separated into 'liquids' and 'solids' by crystallizing three times from acetone at −31°, once from 8 vol., once from 14 vol. and once from 15 vol. The 'solid' acids (1673·0 g.) were converted into methyl esters (1746·5 g.), by the method described by Hilditch (1956), of which 1738·5 g. was fractionally distilled *in vacuo* in a glass Vigreux column (460 cm. × 5 cm.) and yielded 23 fractions and a residue. The twenty-third (wt. 68·30 g.; m.p. 36·4–37·3°; saponification equiv. 298·5; iodine value 9·2) is the main fraction pertaining to this paper. This fraction (65·6 g.) was then submitted to repeated low-temperature crystallization from acetone (and once from ether) at −40°. Data for the first eight crystallizations are recorded in Table 1. In each case shown, the soluble fraction was removed and the insoluble fraction recrystallized.

The following fractions soluble in acetone at −40° (see Table 1) were combined: wt. 1·19 g., m.p. 24·0–24·6°; wt. 0·77 g., m.p. 23·3–24·2°; wt. 0·42 g., m.p. 27·0–27·7°. After conversion into fatty acids (wt. 1·97 g., m.p. 58·2–59·7°) this fraction was crystallized five times from acetone at −13°, the first two crystallizations being from 20 vol., the second two from 40 vol. and the final from 60 vol. The final insoluble fraction (wt. 1·23 g.; m.p. 62·7–63·1°; iodine value 67·0) was further purified by elution of its methyl esters through a column (60 cm. × 2·5 cm.) of activated silicic acid (75 g.) prepared according to the method of Hirsch & Ahrens (1958). Light petroleum (b.p. 40–60°) was used as eluent for the first 15 fractions, light petroleum

containing 1% of ether for the next four fractions and light petroleum containing 5% of ether for the final fraction (see Table 2). The nineteenth fraction (wt. 0·73 g., m.p. 28·3–28·9°) when converted into fatty acids possessed the following chemical and physical properties, which identified it as *trans*-octadec-16-enoic acid: m.p. 65·6–66·2° [Kapp & Knoll (1943) reported m.p. 62·8–63·5° for synthetic *trans*-octadec-16-enoic acid]; saponification equiv. 281·8 (calc. for $C_{18}H_{34}O_2$: 282·5); combustion analysis (Found: C, 76·8; H, 12·5. Calc. for $C_{18}H_{34}O_2$: C, 76·5; H, 12·1%); oxygen analysis (Found: O, 11·25%. Calc. for $C_{18}H_{34}O_2$: 11·4%); iodine value 86·8 (calc. for *n*-octadecenoic acid: 89·8); X-ray long spacing, 44·6 Å, when standardized against pure stearic acid 40·1 Å [Swern, Witnauer, Fusari & Brown (1955) reported 44·9 Å for *trans*-octadec-6-enoic and -8-enoic acid and 45·5 Å for *trans*-octadec-10- and -12-enoic acid: for each of the odd-numbered *trans*-octadec-7-, -9- and -11-enoic acids they reported 49·0 Å]. Methyl esters: m.p. 28·3–28·9°; hydrogenation of 0·10 g. of methyl esters with platinum oxide as catalyst, followed by hydrolysis, yielded 0·09 g. of fatty acids with m.p. 68·0–69·0° and when mixed in equal proportions with pure stearic acid (m.p. 69·9–70·2°) gave m.p. 69·0–69·6°.

*Gas–liquid-chromatographic analysis of methyl esters.* With the 20% polydiethylene glycol adipate column, one major peak was recorded with $R_F$ 1·27, representing approx. 99% of the total peak areas, and two minor peaks of similar area, one with $R_F$ 1·00 (corresponding to methyl stearate or methyl elaidate) and one with $R_F$ 1·35. With the 5% Apiezon L column, with added methyl stearate only one peak appeared.

*Infrared analysis.* With a Perkin–Elmer model 21 spectrophotometer, a solution containing 15 mg. of methyl esters/ml. of carbon disulphide solution was examined in a 0·0846 cm. cell and the *trans* peak was found to absorb at 966 cm.$^{-1}$. The *trans* peak of a sample of methyl elaidate absorbed at 964 cm.$^{-1}$. On comparing solutions of the same concentration, the intensity of the *trans* peak was 1·16 times the intensity of the corresponding peak of methyl elaidate.

*Location of the double bond.* By the method of Armstrong

Table 1. *Low-temperature crystallization of methyl ester fraction*

All crystallizations were at −40°.

| | | Soluble | | Insoluble | |
|---|---|---|---|---|---|
| Wt. (g.) | Conditions of crystallization | Wt. (g.) | M.p. | Wt. (g.) | M.p. |
| 65·60 | Acetone, 40 vol. | 3·90 | 10·8–12·8° | 61·70 | 30·0–38·8° |
| 61·70 | Acetone, 40 vol. | 1·39 | 17·2–18·7 | 60·31 | 37·7–38·5 |
| 59·80 | Acetone, 60 vol. | 1·19* | 24·0–24·6 | 58·61 | 37·9–38·8 |
| 58·61 | Acetone, 60 vol. | 0·77* | 23·3–24·2 | 57·84 | 37·6–38·6 |
| 57·84 | Acetone, 100 vol. | 0·69 | 28·8–29·3 | 57·15 | 37·8–39·0 |
| 52·12 | Ether, 40 vol. | 2·40 | 34·4–35·3 | 49·72 | 37·8–38·7 |
| 49·72 | Acetone, 100 vol. | 0·42* | 27·0–27·7 | 49·30 | 38·1–38·9 |
| 49·30 | Acetone, 100 vol. | 0·32 | 34·0–34·6 | 48·98 | 38·0–39·0 |

* These fractions were combined.

Table 2. *Chromatographic separation of methyl esters* (1·25 *g.*) *on silicic acid column*

| | | Eluent | | |
|---|---|---|---|---|
| Fraction | Wt. (mg.) | Light petroleum (ml.) | Ether (%) | M.p. |
| 1 | 10 | 100 | — | 22·0–40·0° |
| 2 | — | 100 | — | — |
| 3 | 1 | 100 | — | — |
| 4 | 5 | 100 | — | 35·0 |
| 5 | 10 | 100 | — | 34·0–35·3 |
| 6 | 60 | 200 | — | 33·4–34·3 |
| 7 | 70 | 200 | — | 35·0–35·5 |
| 8 | 70 | 200 | — | 35·2–35·5 |
| 9 | 40 | 200 | — | 35·2–35·5 |
| 10 | 30 | 200 | — | 33·2–33·6 |
| 11 | 30 | 200 | — | 32·0–32·5 |
| 12 | 20 | 200 | — | 30·1–31·6 |
| 13 | 20 | 200 | — | 30·6–30·9 |
| 14 | 50 | 500 | — | 29·2–29·6 |
| 15 | 30 | 250 | — | 28·8–29·4 |
| 16 | 20 | 200 | 1 | 28·8–29·4 |
| 17 | 20 | 200 | 1 | 28·8–29·4 |
| 18 | 40 | 200 | 1 | 28·3–29·0 |
| 19 | 730 | 300 | 1 | 28·3–28·9 |
| 20 | Trace | 300 | 5 | — |

& Hilditch (1925), as recommended by Haverkamp Begemann, Keppler & Boekenoogen (1950), 0·10 g. of fatty acids was dissolved in 2 ml. of acetic acid and oxidized with 0·38 g. of finely powdered potassium permanganate. After decolorization with sodium metabisulphite in a few drops of 2N-sulphuric acid, the solution was extracted with ether and washed with water. Ether from the ethereal extract was removed by distillation and the solid residue was dried *in vacuo*, giving 0·09 g. of solid material, m.p. 117·0–119·0°, of which a sample of 0·02 g. was converted into methyl esters, and on analysis by gas–liquid chromatography showed one major peak with $R_F$ 4·50 (for purified thapsic acid, observed $R_F$ 4·50), representing 90·3% of the combined peak areas. The remaining 0·07 g. was crystallized successively at room temperature as follows: twice from 50 vol. of ethylene dichloride, once from 50 vol. of light petroleum (b.p. 40–60°), once from 50 vol. of water and once from 100 vol. of water, removing in all only a trace (0·01 g.) of soluble material. Purification of the remaining 0·06 g. of residue was effected by two crystallizations each from 50 vol. of acetic acid at room temperature, yielding a white crystalline insoluble fraction (wt. 0·04 g.; m.p. 123·2–124·0°) (Found: C, 67·3; H, 10·5. Calc. for $C_{16}H_{30}O_4$: C, 67·1; H, 10·6%). When this 0·04 g. fraction was mixed in equal proportions with purified commercial synthetic thapsic (hexadecanedioic) acid (m.p. 123·3–124·0°; saponification equiv. 142·3) it gave a m.p. of 123·1–124·0° and when converted into the dimethyl ester (m.p. 50·9–51·3°) and mixed in equal proportions with the corresponding ester of purified synthetic thapsic acid (m.p. 51·2–51·7°) gave m.p. 51·1–51·3°. Gas–liquid analysis showed it to have one major peak with $R_F$ 4·54 (observed for purified dimethyl ester of synthetic thapsic acid, $R_F$ 4·50) representing 98·3% of the combined peak areas. The soluble fractions from this series of crystallizations were combined (wt. 0·03 g.; m.p. 117·0–119·3°) and when converted into dimethyl esters and analysed by gas–liquid chromatography the fraction was shown to be approximately 88·3% of thapsic acid with $R_F$ 4·53.

Four other fractions, combined weight approximately 1 g., which were isolated in this work and which were derived from original fractions 18–23 referred to earlier, consisted wholly or largely of *trans*-octadec-16-enoic acid as determined by melting points, mixed melting points, saponification equivalents, iodine values, and gas–liquid-chromatographic and X-ray analyses.

*Purified synthetic thapsic acid.* In order to provide a standard acid and to establish the identity of the isolated dicarboxylic acid as thapsic acid, a sample of synthetic thapsic acid from L. Light and Co. Ltd., Colnbrook, Bucks., was purified by repeated crystallization from 25 vol. of acetic acid followed by saponification, removal of any unsaponifiable matter, regeneration of the dibasic fatty acids and further crystallization from water. The purified synthetic thapsic acid then possesssed the following constants: m.p. 123·3–124·0° [Ralston (1948) records m.p. 125·0–125·2° for thapsic acid; Backderf (1956) 122·8°]; saponification equiv. 142·3 (calc. equiv. for pure thapsic acid: 143·2); combustion analysis (Found: C, 67·6; H, 10·2. Calc. for $C_{16}H_{30}O_4$: C, 67·1; H, 10·6%); dimethyl ester: m.p. 51·2–51·7° [Ralston (1948) records m.p. 51·6° for dimethyl ester of thapsic acid]. Gas–liquid-chromatographic analysis of this purified synthetic thapsic acid recorded one peak only with $R_F$ 4·50.

## DISCUSSION

The chemical and physical properties of the fraction reported in this paper, supported by identification of thapsic acid as the dicarboxylic acid product of oxidative fission, conclusively establish its identity as *trans*-octadec-16-enoic acid. Although the molecular weight as determined by the saponification equivalent and confirmed by the C, H and O analyses indicates a $C_{18}$ monocarboxylic fatty acid, the iodine value (86·8), which closely corresponds with the theoretical value (89·8), proves the presence of a single double bond. Further proof of unsaturation is provided by the fact that hydrogenation yielded stearic acid. The melting point (65·3–66·1°) of the fatty acid isolated is similar to that (62·8–63·5°) obtained by Kapp & Knoll (1943) for synthetic *trans*-octadec-16-enoic acid, and its closeness to the melting point of stearic acid (69·9–70·2°) distinguishes it from all other known octadecenoic acids. It has been observed by Huber (1951) that the *cis*- and the *trans*-octadecenoic acid series each exhibits an alternation in melting points just as do the *normal* saturated odd- and even-numbered fatty acids. Although the melting points of all the *cis*- and *trans*-octadecenoic acids are not known, the melting point of the fraction reported in this paper conforms readily to the pattern of the even and not to the odd series. This is demonstrated by comparison of the synthesized even-numbered $C_{18}$ *trans* isomers (-6-, m.p. 52·7–53·4°; -8-, m.p. 51·5–52·3°; -10-, m.p. 52·0–52·6°; -12-, m.p. 52·0–53·0°) with those of the odd-numbered homologues: -7-, m.p. 43·5–44·5°; -9-, m.p. 44·5–45·5°; -11-, m.p. 43·5–44·5°. [Melting points for the above synthetic *trans*-octadec-7-, -8-, -9-, -10-, -11- and -12-enoic acids were reported by Huber (1951), whereas that for *trans*-octadec-6-enoic acid was reported by Swern *et al.* (1955).] Similarly, the melting point of this fatty acid distinguishes it from synthetic *trans*-octadec-17-enoic acid [reported: m.p. 55·0–55·5°, Kapp & Knoll (1943); 55·5–56·1°, Huber (1951)], which has a double bond in the terminal position in the chain. Consistent with this acid's being an even-numbered *trans*-octadecenoic acid is the fact that its melting point contrasts with the considerably lower melting points of the corresponding *cis*-octadecenoic acid homologues, as for example *cis*-octadec-6-enoic acid, m.p. 30·0–30·2° (Fusari, Greenlee & Brown, 1951), *cis*-octadec-9-enoic acid, m.p. 13·3–13·5° (Fusari *et al.* 1951), *cis*-octadec-11-enoic acid, m.p. 13·5–14·5° (Bounds, Linstead & Weedon, 1954), and *cis*-octadec-12-enoic acid, m.p. 26·8–27·6° (Huber, 1951). Positive identification of this fraction as a *trans* acid was provided by infrared-spectrophotometric analysis of its methyl esters. Just as the odd- and even-numbered *trans*-

octadecenoic acids display an alternation of melting points, so also do they exhibit an alternation in X-ray long spacings (Lutton & Kolp, 1951). The measurement reported in this paper for the isolated fatty acid is 44·6Å, and this value is consistent with its being an even-numbered *trans*-octadecenoic acid isomer [Lutton & Kolp (1951) record 45·5Å for *trans*-octadec-6-, -8-, -10- and -12-enoic acids] and incompatible with its being an odd-numbered isomer [Lutton & Kolp (1951) record 49·5Å for *trans*-octadec-7-, -9- and -11-enoic acids].

Proof of the position of the double bond in the fatty acid fraction being between the sixteenth and seventeenth carbon atoms was provided by the identification of thapsic acid as the dicarboxylic acid produced on oxidation with potassium permanganate and acetic acid. The oxidation of 0·10 g. of fatty acids yielded 0·09 g. of solid dicarboxylic acids, m.p. 117–119°, and gas–liquid-chromatographic analysis of a sample showed it to contain 90·3% of thapsic acid (i.e. 0·08 g.), assuming the amount detected corresponded with the amount applied to the column. The experimental yield of thapsic acid was thus 80% of theoretical. The presence of a preponderance of thapsic acid in the products resulting from the oxidation of this fatty acid is confirmed, independently of gas–liquid chromatography, by its isolation in the pure state (0·04 g.; m.p. 123·2–124·0°) by crystallization from solvents.

Based on the weight of the fractions isolated in this work together with the amounts estimated to be present in accompanying impure fractions, it is estimated that the content of *trans*-octadec-16-enoic acid in the sample of summer butterfat under investigation represents approximately 0·2% of the total weight of fatty acids. Backderf (1956) and Backderf & Brown (1958), however, believed this acid to be present in their summer sample to the extent of 1–2% of the total butterfat fatty acids.

The occurrence of *trans* acids in butterfat appears to be associated with two significant factors: (1) in ruminants dietary fats are subject to hydrogenation by rumen micro-organisms (Reiser, 1951; Hartman, Shorland & McDonald, 1954, 1955; Shorland, Weenink & Johns, 1955; Reiser & Reddy, 1956; Shorland, Weenink, Johns & McDonald, 1957); (2) partial hydrogenation of unsaturated fatty acids is accompanied by migration of the double bond in either direction and by the formation of positional and geometrical isomers (Allen & Kiess, 1955; Rebello & Daubert, 1951; Feuge & Cousins, 1960). As the diet of ruminants is normally pasture, the fatty acids of which are mainly linolenic acid with lesser amounts of linoleic and oleic acids (Shorland, 1941, 1953; Hilditch & Jasperson, 1945), it is feasible that a wide range of positional and geometrical isomers of the $C_{18}$ unsaturated acids should be present in ruminant body and milk fats.

## SUMMARY

1. *trans*-Octadec-16-enoic acid has been isolated from butterfat and characterized.

2. The weight of *trans*-octadec-16-enoic acid isolated from the sample of summer butterfat investigated represented approximately 0·2% of the total weight of fatty acids.

3. This acid has not formerly been isolated from a natural fat and characterized, although its presence in butterfat was deduced by Backderf (1956) and Backderf & Brown (1958), who conclusively identified thapsic acid as the main dicarboxylic component when appropriate $C_{18}$ *trans* concentrates were oxidized with potassium permanganate and acetic acid.

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# Metabolism of Nitrate by Cattle

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Nitrate toxicity has been observed frequently in ruminants fed on a variety of plant materials high in nitrate (Bradley, Beath & Eppson, 1939; Grisby & Farwell, 1950; Newson, Stout, Thorp, Barber & Groth, 1937; Savage, 1949; Whitehead, Viets & Moxon, 1948; Whitehead & Moxon, 1952). Davidson, Doughty & Bolton (1941) described the toxicity symptoms in nitrate poisoning in detail. Bradley *et al.* (1939) indicated that the symptoms of nitrate toxicity are associated with the high levels of methaemoglobin and hence the low oxygen-carrying capacity of the blood of affected animals. Lewis (1951) demonstrated that micro-organisms in the rumen of sheep reduce nitrate to ammonium ions with nitrite as an intermediate; enough nitrite was transferred to the blood to convert a considerable fraction of the haemoglobin into methaemoglobin. Sund, Wright & Simon (1957) reported that a non-contagious abortion observed among cattle pastured on marsh land in central Wisconsin apparently was a manifestation of nitrate toxicity.

The work presented here was designed to study the time-course of nitrate reduction in the rumen, the changes induced in the blood by metabolites of nitrate, and the disposition of [$^{15}$N]nitrate by the animal.

## MATERIALS AND METHODS

The experimental animals were 700–800 lb. Hereford cows with permanent rumen fistulas which were fitted with a plug. A known amount of $KNO_3$ in 500 ml. of water was added through the rumen fistula each morning before the cow was offered hay, concentrates and water *ad lib.* The conversion of haemoglobin into methaemoglobin in the blood was determined daily about 3 hr. after the administration of $KNO_3$. In the experiments with $^{15}N$, a solution of $K^{15}NO_3$ was added to the rumen through the fistula in a cow which showed about 15% methaemoglobin in its daily samples of blood. Throughout the day that $K^{15}NO_3$ was added, periodic samples were taken of rumen liquor, blood and urine. Blood samples were removed from the jugular vein. Analysis for methaemoglobin was performed immediately on freshly drawn blood, and the remaining blood was treated with sodium oxalate to prevent clotting. Urine samples obtained with a catheter were preserved with toluene in a refrigerator until analysed. The rumen liquor withdrawn through the fistula was strained through 2 layers of cheese cloth; it then was clarified by the method of Lewis (1951), and the clear supernatant solution was used for analysis.

Analyses for nitrate, nitrite, urea and ammonia in the rumen liquor, blood and urine were made by the methods described in the A.O.A.C. handbook (Lepper, 1945). $^{15}N$ was determined with a Consolidated–Nier isotope ratio mass spectrometer. Methaemoglobin and total haemoglobin were determined by the method of Evelyn & Malloy (1938). The blood sample treated with oxalate was centrifuged, and the cells were washed twice by centrifuging from 0·85% NaCl and then were refrigerated until used. Samples (10 ml.) of the supernatant fluid were stirred while to each were added 3 ml. of 0·5M-$Ba(OH)_2$ and 3 ml. of 0·5M-$ZnSO_4$ to precipitate the proteins. The precipitate was removed by centrifuging, and the clear supernatant fluid was used for analysis of ammonium, nitrite and nitrate ions.

Haemin and globin were separated and purified essentially as described by London, Morell & Kassenaar (1958).

The washed red cells were lysed in cold deionized water, and the lysed suspension was filtered with stirring into 12 vol. of chilled acetone containing 1·2 ml. of 12 N-HCl/100 ml. The precipitated globin was filtered and then washed with chilled acetone. The washings were added to the filtrate carrying the haemin in solution. The globin was purified by dissolving the crude precipitate in water, adding 14 % trichloroacetic acid to complete precipitation, and washing the new precipitate twice with 7 % trichloroacetic acid and once with distilled water. The globin was dissolved in N-NaOH, and the precipitation and washings were repeated. The precipitate finally was washed once with a mixture of equal volumes of acetone and ether, twice with acetone and once with ethanol before drying. The dried white powder was hygroscopic.

The haemin was recovered from the acetone solution by addition of one-third volume of deionized water followed by vacuum evaporation, filtration and washing with two portions of cold water. It was purified further by dissolving it in acetone and repeating the precipitation and washings before drying it over $CaCl_2$ in a vacuum.

## RESULTS

### *Metabolism of nitrate added directly to the rumen*

The concentrations of ammonium, nitrite and nitrate ions in the rumen liquor were followed periodically in a cow given 70 g. of $KNO_3$ through a rumen fistula. The nitrate concentration decreased rapidly whereas ammonium and nitrite increased (Fig. 1). Ammonium and nitrite ions were at their maximum concentrations about 3 hr. after nitrate was administered.

Although rather high concentrations of nitrite were produced in the rumen, very little methaemoglobin was detected in the blood, and appreciable concentrations appeared only after several days of treatment (Table 1). After 16 days, the daily dose of $KNO_3$ was increased to 100 g. As indicated in Table 1, the percentage conversion into methaemoglobin approached 15 % after a few days on 100 g. of $KNO_3$ a day. In later experiments, the animals were 'conditioned' by giving them 100 g. of $KNO_3$ daily until their blood carried about 15 % methaemoglobin 3 hr. after treatment.

Experiments were performed with $K^{15}NO_3$, so that the nitrate nitrogen and products derived from it could be traced. A solution of 120 g. of $K^{15}NO_3$, 1·7 atom % $^{15}N$ excess, was introduced into the rumen through the fistula of a 'conditioned' cow in the morning before feeding. Analyses from the first experiment are recorded in Figs. 2 and 3. Fig. 2 indicates the accumulation of methaemoglobin in the blood of the treated cow and the $^{15}N$ concentrations of the nitrate plus nitrite fraction and the ammonia fraction in the rumen liquor. A peak value of 16 % for blood methaemoglobin was recorded 3 hr. after labelled nitrate was supplied; this peak appeared about an hour after the peak concentration of $^{15}N$ in the ammonium

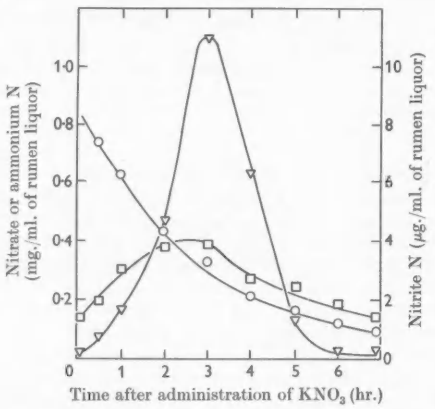


Fig. 1. Reduction of nitrates in the rumen of a cow receiving 70 g. of $KNO_3$ via rumen fistula. ○, Nitrate nitrogen; ▽, nitrite nitrogen; □, ammonium nitrogen.

Table 1. *Induction of methaemoglobin formation in the blood of cattle by repeated dosing with potassium nitrate*

| Days of treatment* | Time after dosing (hr.) | Percentage conversion into methaemoglobin | Days of treatment† | Time after dosing (hr.) | Percentage conversion into methaemoglobin |
|---|---|---|---|---|---|
| 0 | 0 | 0·26 | 16 | 6 | 0·62 |
| 1 | 0 | 0·25 | 19 | 6 | 2·44 |
| 1 | 4 | 0·20 | 20 | 4 | 11·00 |
| 3 | 4 | 1·20 | 20 | 6 | 1·70 |
| 4 | 6 | 0·00 | 23 | 3·5 | 10·50 |
| 5 | 2 | 0·00 | 23 | 5 | 7·80 |
| 5 | 4 | 1·20 | 23 | 6 | 6·40 |
| 5 | 6 | 0·30 | 25 | 3 | 14·30 |
| 9 | 4 | 4·65 | 27 | 3 | 14·90 |
| 12 | 5 | 1·87 | 28 | 4 | 14·20 |
| 12 | 6 | 1·68 | — | — | — |

* Animal received 70 g. of $KNO_3$ daily.
† Animal was shifted to 100 g. of $KNO_3$ on day 16 and received this amount daily thereafter.

ions of the rumen was reached. The decrease in $^{15}N$ concentration in the nitrate plus nitrite fraction followed a more precipitous curve than that observed earlier for the disappearance of unlabelled nitrate from the rumen (Fig. 1).

Fig. 3 indicates the appearance of $^{15}N$ in the fractions recovered from urine; the curve for methaemoglobin formation in the blood is included for reference. The total nitrogen carried a higher concentration of $^{15}N$ and reached a peak concentration of $^{15}N$ sooner than urea or ammonium nitrogen. Urea and ammonium ions reached their highest concentrations of $^{15}N$ at almost the same time; the urea had the higher concentration of $^{15}N$.

The blood samples in the first experiment were too small to provide sufficient nitrogen for proper mass analysis of fractions; the protein nitrogen diluted the $^{15}N$ excessively in the total blood nitrogen. Adequate samples of blood (125 ml.) for mass analysis on fractions were taken in the second experiment. Fig. 4 shows the $^{15}N$ concentrations in various nitrogenous fractions from the blood of a cow that received $K^{15}NO_3$ in Expt. 2. $^{15}N$ accumulated quickly in the nitrate plus nitrite fraction of the blood and remained high during the 8 hr. period of sampling (note that these values in Fig. 4 are divided by 10). The methaemoglobin level approached a maximum of 22 % at 3 hr. The highest concentration of $^{15}N$ in the ammonium ions in the blood also was observed near 3 hr., when its atom % $^{15}N$ excess was about a twelfth of that in the nitrate plus nitrite fraction. The $^{15}N$ in haemin reached a peak concentration more rapidly than did the $^{15}N$ of the ammonium ion. The globin fraction showed no detectable increase in $^{15}N$ above its normal abundance, although the haemin

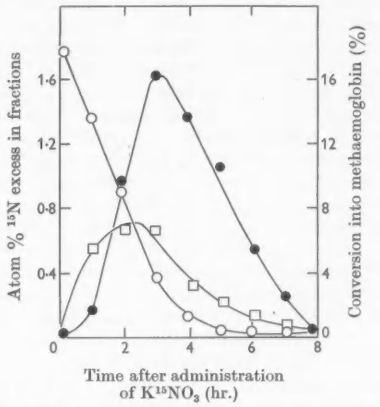


Fig. 2. Reduction of nitrate in a cow's rumen and formation of methaemoglobin in the blood. 120 g. of $K^{15}NO_3$, 1·7 atom % $^{15}N$ excess, was added to the rumen. Expt. 1: ○, nitrate nitrogen plus nitrite nitrogen; □, ammonium nitrogen; ●, percentage conversion into methaemoglobin.

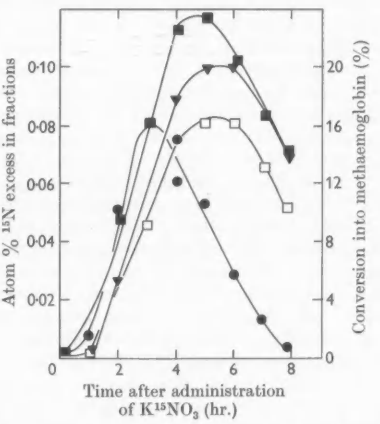


Fig. 3. Formation of methaemoglobin in the blood and accumulation of $^{15}N$ in the nitrogenous compounds of the urine of a cow receiving 120 g. of $K^{15}NO_3$, 1·7 atom % $^{15}N$ excess, through a rumen fistula. Expt. 1: □, ammonium nitrogen; ▼, urea nitrogen; ■, total nitrogen; ●, percentage conversion into methaemoglobin.

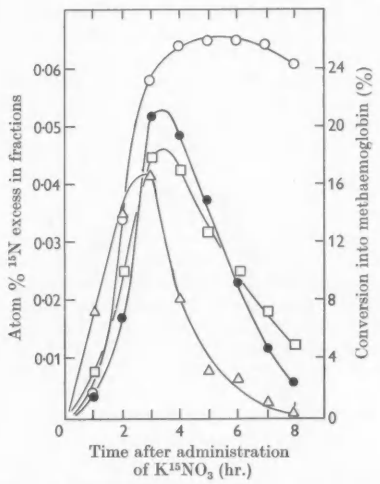


Fig. 4. Formation of methaemoglobin in the blood, and accumulation of $^{15}N$ in the nitrogenous fractions of the blood of a cow receiving 120 g. of $K^{15}NO_3$, 1·7 atom % $^{15}N$ excess, through a rumen fistula. Expt. 2: ○, atom % $N^{15}$ excess $\times 10^{-1}$ of nitrate plus nitrite nitrogen in plasma; □, ammonium nitrogen in plasma; △, haemin; ●, percentage conversion into methaemoglobin.

retained its label despite the vigorous treatment involved in its separation and purification.

The results of urine analysis are presented in Fig. 5. Again the $^{15}N$ in the total nitrogen (which includes nitrate and nitrite) rose more rapidly and to a higher level than in urea or ammonium nitrogen. The concentrations of $^{15}N$ in the total, urea and ammonium ion fractions were highest near 4 hr. and declined to only about half their peak concentrations by 8 hr. The maximum conversion of haemoglobin into methaemoglobin in the blood occurred shortly before the maximum concentration of $^{15}N$ was reached in the urine nitrate.

*Reactions of nitrate and nitrite with blood*

As the haemin from blood of cows given $K^{15}NO_3$ accumulated $^{15}N$, it appeared worth while to study the binding of nitrite *in vitro*. To 5 ml. of fresh blood plus oxalate was added 750 $\mu$g. of $^{15}NO_2^-$, 3·5 atom % $^{15}N$ excess, and the mixture was refrigerated overnight. The methaemoglobin concentration was determined, and the cells were separated and washed by centrifuging until the washings gave a negative test for nitrite. The globin and haemin were separated as described, samples of each were digested and their $^{15}N$ concentrations were determined. The blood containing 51 % methaemoglobin showed no detectable $^{15}N$ in its globin, but the haemin had 0·023 atom % $^{15}N$ excess. This demonstrated that the nitrogen from nitrite was bound firmly enough to the haemin of methaemoglobin to withstand displacement by acetone containing 1·2 ml. of 12 N-HCl/100 ml. The nature of this binding is not known, and no attempt was made to determine whether it was accompanied by an alteration in the composition of the haemin molecule.

Whole blood, cells suspended in 0·85 % NaCl, and lysed cells, each containing about 50 % methaemoglobin, were shaken overnight in pure $O_2$ in an effort to displace nitrite. Although the percentage of methaemoglobin decreased in each case, no detectable nitrite or $NH_3$ was released into the medium. Treatment of other samples of the same materials with 2·5 % neutralized NaCN was followed immediately by disappearance of methaemoglobin, but no significant quantities of nitrite or $NH_3$ appeared in the medium.

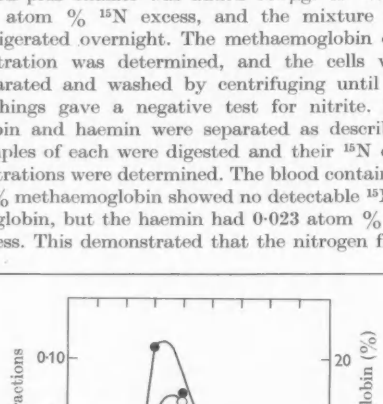


Fig. 5. Formation of methaemoglobin in the blood, and accumulation of $^{15}N$ in the nitrogenous fractions of urine, from a cow receiving 120 g. of $K^{15}NO_3$, 1·7 atom % $^{15}N$ excess, through a rumen fistula. Expt. 2: ○, nitrate nitrogen; □, ammonium nitrogen; ▼, urea nitrogen; ■, total nitrogen; ●, percentage conversion into methaemoglobin.

## DISCUSSION

It is generally recognized that the toxicity of nitrate for ruminants depends upon its reduction to nitrite in the rumen and the combination of the nitrite with haemoglobin to form methaemoglobin in the blood. In addition to the acute symptoms that accompany nitrite poisoning, other conditions, such as a non-contagious abortion in cattle (Sund *et al.* 1957), have been attributed to ingestion of materials high in nitrates. The current studies re-emphasize the very rapid conversion of nitrate into nitrite and ammonium ions in the rumen. The dense microbial flora of the rumen maintains reducing conditions and can reduce added nitrate without delay. Fig. 1 shows the rapid disappearance of nitrate added directly into the rumen of a cow through a fistula, a disappearance which was accompanied by appearance of a maximum concentration of ammonia and nitrite within 3 hr. Our approximations indicated that at this time about 14 % of the added nitrate had been converted into nitrite, but such calculations are subject to substantial corrections for normal pools and absorption of nitrate, nitrite and ammonia.

Despite the fact that nitrite was formed quickly in the rumen with no evident need for adaptation of the microflora, toxicity to the animal was delayed and very little methaemoglobin appeared in the blood for several days after daily additions of nitrate to the rumen were initiated. The nature of this adaptation is not clear. Considerable quantities of nitrate appeared in the blood (Fig. 4), but it seems questionable that substantial reduction of nitrate is accomplished in the blood. One cannot ignore the fact that the time of highest conversion of nitrate into nitrite in the rumen (Fig. 1) very nearly coincided with the time of maximum methaemoglobin formation in the blood

(Fig. 2). Further, the concentration of nitrate persisted in the blood (Fig. 4) beyond the time of a precipitous decrease in percentage of methaemoglobin and the similar decrease in nitrite concentration in the rumen. Data still are insufficient to account accurately for the amount of nitrite formed in the rumen and in the blood, but Pfander, Garner, Ellis & Muhrer (1957) found very little toxicity from nitrate added intravenously, an observation that supports the primary formation of nitrite in the rumen rather than in the blood.

[$^{15}$N]Nitrate was added directly into the rumen to yield more detailed and specific information on nitrate reduction in the rumen, the transfer of nitrate and its products to the blood stream, and their elimination in the urine. The $^{15}$N concentration of the nitrate plus nitrite fraction decreased rapidly (Fig. 2). The $^{15}$N of the ammonium fraction increased as that of the nitrate plus nitrite decreased. After about 2·5 hr., the $^{15}$N concentration of the ammonium ions remained higher than that of the nitrate plus nitrite ions. This response likely arose from a differential dilution of the ions; the ingested feed carried much more nitrate than it did nitrite or ammonia, so that dilution of the $^{15}$N in ammonia occurred more slowly than the dilution in the nitrate plus nitrite.

The maximum concentration of nitrite in the rumen appeared about 3 hr. after nitrate was added to the rumen (Fig. 1), and the maximum conversion of haemoglobin into methaemoglobin occurred at almost the same time (Fig. 2). One would expect very little delay in methaemoglobin formation once the nitrite reached the blood stream. The data support the idea that the bulk of the nitrite (and by implication ammonia and nitrate) is absorbed directly into the blood from the rumen rather than from lower sections of the digestive tract. The virtual coincidence of attaining a maximum nitrite concentration in the rumen and a maximum methaemoglobin level in the blood indicates that the absorption is very rapid.

In relation to the rapid drop in total nitrate nitrogen in the rumen (Fig. 1) and $^{15}$N in the nitrate plus nitrite of the rumen (Fig. 2) it is interesting to observe (Fig. 4) the persistence of a high concentration of $^{15}$N in the nitrate plus nitrite fraction in the blood plasma 4–8 hr. after the administration of potassium [$^{15}$N]nitrate. The rapid decrease in the percentage methaemoglobin after 3–3·5 hr. (Figs. 2–4) suggests that the nitrate plus nitrite fraction of the plasma consisted mostly of nitrate, and that the conversion of nitrate into nitrite in the blood probably was negligible.

The $^{15}$N concentration of the ammonium ions always was far less than that of the nitrate plus nitrite of the plasma (Fig. 4), although they were approximately equivalent in the rumen after 2·5 hr. (Fig. 2). More favourable absorption of nitrate plus nitrite than of ammonium ion from the rumen into the blood, or more extensive isotopic dilution of ammonium than of nitrate plus nitrite in the blood, could explain the observations.

The peak concentration of $^{15}$N in the haemin appeared before the maximum total conversion into methaemoglobin (Fig. 4). This suggests that the $^{15}$N concentration of the nitrite in the rumen was at a maximum before the absolute concentration of nitrite in the rumen reached a maximum under our experimental conditions. It again emphasizes the rapidity with which nitrite is absorbed from the rumen into the blood. Land & Virtanen (1959) reported incorporation of $^{15}$N from ammonium nitrate into milk protein an hour after feeding with the labelled compound.

The appearance of maximum concentrations of $^{15}$N in the total nitrogen fraction of the urine was earlier than for the urea and ammonium nitrogen (Fig. 3) probably because the total nitrogen included nitrate. The concentrations of $^{15}$N in urea and ammonium ions rose and fell together and reached peak values at approximately the same time (5–6 hr. after administering potassium [$^{15}$N]-nitrate). Fig. 5 emphasizes that the highest concentration of $^{15}$N in nitrate appeared near the time of greatest conversion of haemoglobin into methaemoglobin. Again these appeared before the highest concentrations of $^{15}$N appeared in the total nitrogen, urea nitrogen and ammonium nitrogen. Comparison of Figs. 3–5 suggests that there was a lag of as much as an hour between the times the highest concentrations of $^{15}$N were observed in the urine compared with the blood. In part, this may reflect inherent delay in sampling the urine rather than a true lag in equilibration between the two fluids.

## SUMMARY

1. A cow receiving a direct addition of 70–100 g. of potassium nitrate a day into the rumen through a fistula showed no methaemoglobin in the blood for several days. This delay in methaemoglobin formation could not be attributed to failure of the micro-organisms of the rumen to form nitrite, for they immediately showed vigorous nitrate reduction.

2. Potassium [$^{15}$N]nitrate was added to the rumen to trace the formation, absorption and elimination of nitrate and the compounds formed from it. Nitrate, nitrite and ammonium ions were absorbed in considerable quantities directly from the rumen into the blood. The highest concentrations of $^{15}$N in the ammonium ion in the blood appeared 3–4 hr. after potassium [$^{15}$N]nitrate was added to the rumen, whereas the highest $^{15}$N concentration in the nitrate plus nitrite fraction

appeared in the blood after 5–6 hr., and the high concentration persisted.

3. The curves for excretion of the $^{15}N$-labelled total, nitrate, urea and ammonium nitrogen fractions in the urine lagged behind their corresponding curves for appearance and disappearance of these components in the blood by about an hour.

4. The formation of methaemoglobin in the blood followed the time course of nitrite formation in the rumen rather closely, suggesting that nitrite was passed rapidly and directly from the rumen to the blood. There was no direct evidence for nitrite formation from nitrate in the blood.

5. Nitrite was bound tightly to the haem of methaemoglobin, for it could be recovered in haem that had been subjected to treatment with acetone containing 1·2 ml. of 12 N-hydrochloric acid/100 ml.

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# Plant Polyphenols

## 4. HYDROXYCINNAMIC ACID–SUGAR DERIVATIVES*

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Hydroxycinnamic acids, which occur in most higher plants, are almost always present in bound form and are liberated from plant extracts by hydrolysis with acid or alkali. Of the organic substances known to occur in combination with hydroxycinnamic acids, quinic acid is the one most frequently mentioned in the literature: thus chlorogenic acid (a quinic ester of caffeic acid) appears to be almost ubiquitous and at least four other caffeoyl quinic acids are known. *p*-Coumaroylquinic acid is also reported to occur with some frequency (Cartwright, Roberts, Flood & Williams, 1955; Griffiths, 1959). In contrast, few simple sugar derivatives have been described (Karrer, 1958) and, with the exception of *o*-coumaroylglucoside (melilotoside) (Charaux, 1925), none has been fully characterized.

In view of current interest in the biosynthesis (McCalla & Neish, 1959), natural distribution (Bate-Smith, 1954; Herrmann, 1958) and biological function (Lee & Le Tourneau, 1958; Rabin & Klein, 1957) of the hydroxycinnamic acids, a study was made of their derivatives occurring in the potato and a number of other higher plants. Besides the *p*-coumaroylglucose and caffeic acid 3-$\beta$-glucoside found in potato berries (Corner & Harborne, 1960), seven other naturally occurring cinnamic acid–glucose derivatives have been found. Further, simple glucose esters have been identified in nearly a hundred plant species.

The finding that glucose esters are of wide occurrence suggests that they may be formed during the metabolism of free hydroxycinnamic acids in plant tissues. Previous work has only indicated that phenolic glucosides (Kosuge & Conn, 1959) and quinic esters (Levy & Zucker, 1960) are

* Part 3: Harborne & Sherratt (1961).

formed *in vivo* from the free acids. A number of these acids therefore were fed to a variety of plants and the metabolites isolated and identified. The results, which have already been reported briefly (Harborne & Corner, 1960), are now presented in detail.

## MATERIALS AND METHODS

*Hydroxycinnamic acids and derivatives.* The free acids were obtained from L. Light and Co. Ltd., or from Fluka A.G., Buchs, Switzerland, or were synthesized by standard procedures from malonic acid and the appropriate aldehyde (Johnson, 1942); they were recrystallized twice from water or aqueous ethanol. The methyl esters were prepared by heating the corresponding free acid in methanol containing 5% (v/v) of sulphuric acid for 2 hr. on a steam bath. Chlorogenic and *iso*chlorogenic acid were obtained by purchase; *p*-coumaroylquinic acid was supplied by A. H. Williams, Long Ashton Research Station. Melilotoside was synthesized from helicin and malonic acid (Helferich & Lutzmann, 1938).

*Sugars and enzymes.* Rutinose was prepared from rutin by hydrolysis with 10% (v/v) acetic acid soln. for 4 hr. at 100°. Gentiobiose and cellobiose were obtained commercially. The purified β-glucosidase, as supplied by L. Light and Co. Ltd., did not contain any free sugars or other contaminants of low mol. wt. and at a concn. of 0·5 mg./ml. hydrolysed aesculin (at a concn. of 5 mg./ml.) to aesculetin at pH 4·6 and 37°. A sample of anthocyanase, prepared from *Aspergillus niger* and supplied by Rohm and Haas Co., Philadelphia, U.S.A., was used as a source of esterase. The enzyme mixture (at a concn. of 7 mg./ml.) completely hydrolysed chlorogenic acid (at a concn. of 3 mg./ml.) to caffeic acid and quinic acid in 30 min. at 37° and pH 3·9.

*Paper chromatography and spectroscopy.* Whatman no. 1 and no. 3 papers (chromatography grade) were used. The solvent mixtures employed were: *A*, butan-1-ol–acetic acid–water (4:1:5, by vol.; upper phase); *B*, water; *C*, butan-1-ol–2N-ammonia (1:1, v/v; upper phase); *D*, butan-1-ol–ethanol–water (4:1:2·2, by vol.). Chromatograms were examined in u.v. light of 253 mμ and > 300 mμ sources, in the presence or absence of ammonia vapour.

Spectra were determined with a Unicam SP. 500 spectrophotometer. In measuring the spectra of compounds, eluted from paper chromatograms, allowance for filter-paper impurities was made by using eluates of an appropriate paper blank.

*Feeding experiments.* Leaves were cut from the stems of young plants and the petioles were placed immediately in saturated aqueous solutions of the appropriate cinnamic acids, previously adjusted to pH 7 by the addition of 2N-sodium hydroxide. The leaves were placed, suitably supported, in a controlled environment room at 23° under light-intensity of 800 ft.-candles, applied for 12 hr./day for 1–7 days. Equal numbers of leaves were placed at the same time in distilled water and then treated similarly. Potato disks, 2 mm. thick and 12 mm. in diameter, were cut from scrubbed, freshly harvested tubers of *Solanum tuberosum* cv. 'King Edward', and then washed for 2 hr. with tap water. After drying on filter paper, the disks were placed in Petri dishes containing filter papers soaked in either the aqueous cinnamic acid solutions or in distilled water. The dishes were covered and left at room temp. for 3 days.

After the treatments the plant material was washed with water, dried, cut into small pieces and then plunged into boiling ethanol. After 30 min., the ethanolic extract was decanted and the residue was re-extracted for 30 min. with an equal volume of ethanol. The combined extracts were concentrated *in vacuo* at about 50° and stored overnight at 0° to allow the chlorophyll of green tissue or the starch of tuber tissue to separate out. The clear supernatant liquid was placed as streaks on no. 3 filter paper, which was developed with solvents *A* or *C*. Metabolic products of the cinnamic acids were detected by comparing chromatograms prepared from leaves fed with cinnamic acids with those from leaves fed with water. All new bands on the former chromatograms were cut out, eluted and purified by successive chromatography in solvents *A*–*D*. The substances present in these bands were identified by methods described below.

When doubt existed about the novelty of a particular band on the chromatogram in a feeding experiment, a band of the same $R_F$ was cut from the control chromatogram. The two bands were put through the same purification procedure; direct spectral and chromatographic comparison then indicated clearly whether the compound in question was new or not.

*Isolation of cinnamic acid–sugar derivatives.* These were isolated from fresh leaves, flowers or berries of plants known to contain these substances (see Results) by the procedures described above. Usually, the derivatives present in the concentrated plant extracts were separated in solvent *A*, and the individual bands were purified by chromatography in solvent *B* (to separate from flavonoids), in solvent *C* (to separate from quinic esters of cinnamic acids) and then again in solvent *A*.

*Identification of cinnamic acid–sugar derivatives and related compounds.* Known polyphenols and their hydrolysis products were identified by direct comparison with authentic samples (Harborne, 1960*b*). The cinnamic acid–sugar derivatives were recognized as new compounds by comparing their colour reactions, adsorption spectra and $R_F$ values (Table 1) with those of known, naturally occurring cinnamic acid quinic and methyl esters (e.g. chlorogenic acid and methyl *p*-coumarate). They were identified as follows. Pure solutions were treated with: (*a*) 2N-sodium hydroxide at room temp. for 2–3 hr. (under nitrogen, when *o*-dihydroxylic groups were known to be present in the cinnamic acid); (*b*) N-hydrochloric acid at 100° for 0·5 hr.; (*c*) β-glucosidase [0·2 ml. of aqueous 10% (w/v) solution] in citrate–phosphate buffer, pH 4·5, at 37° for 0·5 hr.; (*d*) esterase [1 ml. of aqueous 10% (w/v) solution] in citrate–phosphate buffer, pH 3·9, at 37° for 6 hr. If hydrolysis took place, the solutions were acidified with Amberlite IRC-120 (H) resin, the free cinnamic acids being extracted into ether and identified by standard procedures (Harborne, 1960*b*). The sugars present in the aqueous residues were identified by comparison with authentic compounds by chromatography in three solvent systems, by spectral measurements on the colour complex formed with resorcinol in sulphuric acid and by their behaviour towards glucose oxidase. The diphenylamine spray reagent (Schwimmer & Bevenue, 1956) and paper electrophoresis were used to distinguish between 1:4- and 1:6-linked disaccharides. The aqueous residues were shown to be free

(except for PC3 and CA5) of non-reducing sugars (benzidine spray), amino acids (ninhydrin spray) and quinic acid and other organic acids (bromophenol blue, silver nitrate and periodate–benzidine sprays).

The ratios of cinnamic acid to sugar were determined in the glycosides by the method used for anthocyanins (Harborne, 1960*a*). The positions of the sugar residues in the glycosides were confirmed by methylation with methyl sulphate and potassium carbonate in acetone for 1 hr. at the b.p., and subsequent hydrolysis with 2N-hydrochloric acid for 0·5 hr. at 100°. The methylated cinnamic acids were extracted into ether and identified by standard procedures (see e.g. Dunlap & Wender, 1960).

*Chromatographic survey of cinnamic acid derivatives.* Fresh leaf, berry, petal or root samples were extracted with boiling ethanol for 0·5 hr., and a portion of the extract, after concentration, was placed as a spot on chromatograms, subsequently developed in solvents *A–D*. A cinnamic acid derivative was recorded as present in a particular plant if its colour reactions and $R_F$ values in at least two solvent systems were the same as the authentic compounds, run on the same chromatograms.

## RESULTS

### *Chemical identification*

Fifteen glucose-containing hydroxycinnamic acid derivatives isolated from natural sources or from plants fed on cinnamic acids (for details, see below) have been characterized. Their properties are shown in Table 1. Compounds OC1, MC1, PC1, CA1, FE1, SN1 and T1 all yield on hydrolysis glucose and the relevant cinnamic acid in equimolecular amounts. The following evidence shows that in these seven compounds D-glucose is attached from its 1-position by a $\beta$-linkage to the carboxyl and not to a phenolic group of the parent cinnamic acid: (1) They are all easily hydrolysed by alkali, esterase and $\beta$-glucosidase, the sugar produced being rapidly destroyed by glucose oxidase. (2) Their colour reactions and spectral characteristics closely resemble those of authentic methyl and quinic esters of the corresponding cinnamic acid. For example, their spectra show the large bathochromic shift of 60–65 m$\mu$ typical of cinnamic acid esters on passing from acid to alkaline solution; by contrast, the alkaline spectrum of melilotoside, (*o*-coumaroylglucoside) shows a hypsochromic shift of 7 m$\mu$. (3) Their chromatographic properties when compared (see Table 1) with quinic esters [i.e. lower $R_F$ values in butanol–acetic acid–water (solvent *A*) but higher $R_F$ values in butanol–2N-ammonia (solvent *C*)] and with the free acids ($\Delta R_F$ values are shown in Table 1) are consistent with their proposed structures. (4) Methylation and subsequent hydrolysis of PC1, CA1 and FE1 give the expected fully methylated cinnamic acid (*p*-methoxy- from PC1 and 3:4-dimethoxy- from CA1 and FE1). By contrast, CA2, 3-glucoside of caffeic acid (see below), gives a partially methylated cinnamic acid, isoferulic acid, on similar treatment. Thus OC1, MC1, PC1, CA1, FE1, SN1 and T1 are the simple D-glucose esters of *o*-, *m*- and *p*-coumaric, caffeic, ferulic, sinapic and 3:4:5-trimethoxycinnamic acid respectively. Compound C1 could not be obtained in sufficient quantity for its characterization to be completed. However, on the basis of its recorded properties and its mode of formation (Table 3), it is provisionally identified as 1-cinnamoylglucose.

The substances OC2, PC2 and CA4 are also simple esters of *o*- and *p*-coumaric and caffeic acid respectively, but they all contain two sugar residues (Table 1). On alkaline hydrolysis, PC2 yields a rhamnosylglucose, chromatographically and electrophoretically identical with rutinose; OC2 and CA4 yield a glucosylglucose, identical with gentiobiose. Thus, although the nature of the disaccharide moieties in these compounds has not been rigorously proved, it is almost certain that OC2 is 1-*o*-coumaroylgentiobiose, PC2 is 1-*p*-coumaroylrutinose and CA2 is 1-caffeoylgentiobiose.

CA2 and CA3, like CA1, give equal amounts of caffeic acid and glucose on hydrolysis with acid or $\beta$-glucosidase. Both compounds have different spectral and colour properties from caffeic acid or its esters and, unlike caffeoylglucose, neither is hydrolysed by alkali or esterase. When methylated, and the product hydrolysed, CA2 yields *iso*ferulic acid. CA2 is therefore the 3-$\beta$-glucoside of caffeic acid; and, by elimination, CA3 must be the 4-$\beta$-glucoside. Thus, all the three possible monoglucose derivatives of caffeic acid (CA1, 2 and 3) have been identified.

Two other compounds have been found, each of which contains a cinnamic acid, glucose and an unidentified residue. The first of these is a *p*-coumaric acid derivative (PC3) which on acid hydrolysis gives glucose and an organic acid, which may be quinic acid. PC3 is not a simple ester, however, and must have an unusual structure, since it is not hydrolysed by $\beta$-glucosidase, by esterase or even by alkali. The other compound, CA5, is hydrolysed by acid or alkali to give caffeic acid and glucose. It is not hydrolysed by $\beta$-glucosidase and is clearly different in its chromatographic properties from the simple glucose derivatives (CA1, 2 and 3). Further work on the characterization of these compounds is in progress.

### *Natural occurrence*

Nine cinnamic acid derivatives containing glucose have been found to occur naturally. The plant sources of the seven that have been completely characterized are shown in Table 2. Three of these, 1-*p*-coumaroyl-, 1-caffeoyl- and 1-feruloylglucose, occur frequently. This is apparent from the

Table 1. *Properties of cinnamic acid–glucose derivatives*

For details see text.

| Designation | $R_F$ values* in solvent | | | | $\Delta R_F$ in solvent *A*: sugar ester minus free acid | Colour | | Spectral maxima | | Hydrolysis products and ratios |
|---|---|---|---|---|---|---|---|---|---|---|
| | *A* | *B* | *C* | *D* | | Ultraviolet | Ultraviolet/ ammonia | 95% Ethanol | Sodium ethoxide | |
| PC1 | 0·66 | 0·73, 0·81 | 0·30 | 0·75 | 0·28 | Colourless | Blue | 235, —, 313 | 240, 365 | *p*-Coumaric acid (1·00), glucose (1·10) |
| PC2 | 0·49 | 0·84 | 0·16 | 0·64 | 0·43 | Colourless | Blue | 232, —, 314 | 240, 364 | *p*-Coumaric (0·75), glucose (1·00), rhamnose (1·00) |
| PC3 | 0·63 | 0·90 | 0·00 | 0·27 | — | Colourless | Blue | 218, —, 312 | 235, 365 | *p*-Coumaric, glucose |
| CA1 | 0·51 | 0·61, 0·72 | 0·08 | 0·63 | 0·28 | Blue | Green | 250, 305, 334 | 265, 400 | Caffeic (1·00), glucose (1·10) |
| CA2 | 0·46 | 0·46 | 0·02 | 0·55 | — | Blue | Intense purple | 228, 295, 311 | 235, 335 | Caffeic (1·00), glucose (1·20) |
| CA3 | 0·29 | 0·77, 0·83 | 0·00 | 0·46 | — | Blue | Turquoise | —, 300, 322 | 247, 365 | Caffeic (1·00), glucose (1·10) |
| CA4 | 0·29 | 0·77, 0·83 | 0·03 | 0·46 | — | Blue | Green | 245, 305, 335 | 265, 400 | Caffeic (1·00), glucose (1·88) |
| CA5 | 0·29 | 0·81, 0·88 | 0·00 | 0·18 | — | Blue | Green | 240, 302, 328 | 265, 405 | Caffeic, glucose |
| FE1 | 0·57 | 0·64, 0·73 | 0·19 | 0·68 | 0·31 | Blue | Green | 240, 300, 330 | 250, 382 | Ferulic (1·00), glucose (1·13) |
| SN1 | 0·47 | 0·47, 0·57 | 0·16 | 0·66 | 0·37 | Blue | Green | 238, —, 330 | 255, 397 | Sinapic (1·00), glucose (1·20) |
| T1 | 0·62 | 0·74, 0·83 | 0·45 | 0·83 | 0·25 | Mauve | Mauve | 230, —, 307 | 230, 307 | 3:4:5-Trimethoxycinnamic (1·00), glucose (0·96) |
| OC1 | 0·75 | 0·79 | 0·40 | 0·76 | 0·18 | Yellow | Orange | 228, 277, 325 | 287, 387 | *o*-Coumaric (0·51)†, glucose (1·00) |
| OC2 | 0·45 | 0·87 | 0·12 | 0·53 | 0·48 | Yellow | Orange | 230, 277, 327 | 285, 389 | *o*-Coumaric (0·61)†, glucose (2·00) |
| MC1 | 0·70 | 0·85 | 0·46 | 0·74 | 0·21 | Colourless‡ | Mauve | 233, 277, 312 | 287, 344 | *m*-Coumaric (1·00), glucose (0·98) |
| C1 | 0·79 | 0·89 | 0·65 | 0·79 | 0·14 | Colourless‡ | Colourless‡ | — — — | —, — | Cinnamic, glucose |

* By comparison, *p*-coumaroylquinic acid has $R_F$ values of 0·71, 0·92, 0·09, 0·54, and caffeoylquinic acid (chlorogenic acid) has $R_F$ values 0·59, 0·67 and 0·84, 0·01, 0·50, in solvents *A*–*D* respectively.

† Figures are low, because material is lost by cyclization to coumarin (detected by its odour).

‡ Visible as absorbing spots on a fluorescent background in ultraviolet light of 253 m$\mu$ wavelength.

Table 2. *Occurrence of cinnamic acid–sugar derivatives in Nature*

Key to methods of comparison, where applicable, with authentic material prepared in feeding experiments: 1, co-chromatography; 2, identification of glucose and the corresponding cinnamic acid on β-glucosidase hydrolysis; 3, spectral measurements; 4, measurement of glucose–cinnamic acid ratio.

| Designation | Sugar derivative | Source | Methods of comparison |
|---|---|---|---|
| PC1 | 1-*p*-Coumaroylglucose | *Lathyrus odoratus* (petals and leaves) | 1, 2, 3 |
| | | *Petunia hybrida* (petals) | 1, 2, 3 |
| | | Tuberous *Solanum* spp. (berries, petals and leaves) | 1, 2, 3, 4 |
| | | *Primula obconica* (petals) | 1, 2, 3 |
| | | *Antirrhinum majus* (petals) | 1, 2, 3, 4 |
| | | *Brunfelsia calycina* (petals) | 1 |
| | | *Raphanus sativus* (young leaves) | 1, 2 |
| PC2 | 1-*p*-Coumaroylrutinose | *Lathyrus odoratus* (petals) | — |
| CA1 | 1-Caffeoylglucose | *Petunia hybrida* (petals) | 1, 2, 3 |
| | | *Ranunculus bulbosus* (leaves, petals) | 1 |
| | | Tuberous *Solanum* spp. (leaves) | 1 |
| | | *Clematis lawsoniana* (petals, leaves) | 1, 2, 3, 4 |
| | | *Helleborus foetidus* (leaves) | 1 |
| | | *Datura knightii, D. meteloides* (leaves) | 1 |
| | | *Cestrum newellii* (leaves) | 1 |
| | | *Raphanus sativus* (leaves) | 1, 2, 3 |
| | | *Brunfelsia calycina* (petals, leaves) | 1 |
| | | *Anagallis arvensis* (leaves) | 1, 2 |
| | | *Antirrhinum majus* (petals) | 1, 2, 3 |
| CA2 | Caffeic acid 3-glucoside | Tuberous *Solanum* wild spp. (berries) | 1, 2, 3, 4 |
| CA4 | 1-Caffeoylgentiobiose | *Petunia hybrida* (petals) | — |
| FE1 | 1-Feruloylglucose | *Antirrhinum majus* (petals) | 1, 2, 3, 4 |
| | | *Petunia hybrida* (petals) | 1, 2, 3 |
| | | *Eulalia japonica* (leaves) | 1, 2 |
| | | *Raphanus sativus* (leaves) | 1, 2 |
| | | Tuberous *Solanum* spp. (leaves) | 1 |
| | | *Brunfelsia calycina* (petals) | 1 |
| SN1 | 1-Sinapoylglucose | *Brassica oleracea* (leaves) | 1, 2, 3 |

results presented in Table 2 and also from a chromatographic survey, which was carried out on the leaves and petals of various herbaceous plants grown at this Institute. Of 204 species chosen at random, 43% contained chlorogenic acid, 31% 1-caffeoylglucose, 13% *p*-coumaroylquinic acid and 5% 1-*p*-coumaroylglucose. Although the identification of these substances was based only on chromatographic comparison, the figures for the quinic esters agree with the results of earlier surveys (e.g. Herrmann, 1958) and, together, indicate that sugar esters occur in higher plants with the same order of frequency as the well-known quinic esters.

Of the plant families examined in more detail, two, Solanaceae and Ranunculaceae, appear regularly to contain glucose esters. Thus these substances have been found in the genera: *Brunfelsia, Cestrum, Lycopersicum, Nierembergia, Petunia* and *Solanum* of the Solanaceae; and in *Aconitum, Anemone, Aquilegia, Caltha, Clematis, Delphinium, Helleborus, Pulsatilla* and *Ranunculus* of the Ranunculaceae. Probably, close examination of the cinnamic esters of plants in other families would reveal a similar situation.

Glucose esters often occur in association with quinic and other esters. Thus leaves of the radish, *Raphanus sativus*, contain what may be choline esters of hydroxycinnamic acids [e.g. sinapin, the sinapic acid ester in the seeds of various Cruciferae (Karrer, 1958)], besides the glucose esters reported in Table 2. Also, chlorogenic acid and 1-caffeoylglucose have been found together in many plants, especially in members of the Solanaceae. As might be expected, the glucose ester content of a particular plant depends upon age and environment. Young radish leaves contain considerable amounts of glucose esters, but older leaves lack them completely; and leaves of tuberous *Solanum* species grown in the glasshouse and in the field show variation in the nature and amounts of their sugar esters.

Table 2 shows that a number of plants each contain more than one sugar ester. Thus petals of *Petunia hybrida* cv. 'Flaming velvet' contain 1-*p*-coumaroyl-, 1-feruloyl- and 1-caffeoyl-glucose as well as 1-caffeoylgentiobiose, a result which has been confirmed by the parallel studies of Birkhafer, Kaiser & Kosmol (1960). The three glucose esters also occur together in young radish leaves, in petals of *Brunfelsia calycina* and in petals of *Antirrhinum majus*. The earlier report (Schütte & Böhme, 1958) that quinic esters are present in the petals of the white genotype, *nivea*, of this latter

plant has been shown to be unfounded (Harborne & Corner, 1961).

Phenolic glucosides are much more restricted in their distribution in Nature than the glucose esters. Only one such compound, caffeic acid 3-$\beta$-glucoside, has been found in the present survey and this occurs exclusively in berries of wild tuber-bearing *Solanum* species (i.e. all 19 species examined). It is absent from the berries of the cultivated potato (12 clones of varying ploidy examined) and of non-tuberous *Solanum* plants (11 species examined). In contrast, berries of cultivated potatoes contain a considerable amount of 1-*p*-coumaroylglucose and free 7-hydroxycoumarin (umbelliferone), which are absent or very rare in the wild species. This finding is of considerable taxonomic interest, since it has a bearing on the relationship between wild and cultivated potatoes. Similar chemical differences in polyphenolic content of tubers (Dodds & Long, 1955; Harborne, 1960*a*) and of petal constituents (Harborne, 1960*b* and unpublished work) have been noted.

The natural sources of the incompletely identified glucose derivatives PC3 and CA5 are as follows: PC3 accompanied by some 1-*p*-coumaroylglucose is the major hydroxycinnamic acid derivative in both leaf and petal of the various colour forms of the garden *Geranium*. CA5 occurs with chlorogenic acid in the leaves of the tomato, cv. 'Potentate', of *Solanum neohawkesii* and of *S. canasense*. A number of other cinnamic acid derivatives, probably containing both sugar and other residues, have been noted during this work and it is hoped to examine those occurring in the leaves of wild potato species in due course.

*Formation from cinnamic acids*

The main products of feeding the leaves of a variety of plant species for periods of 1–3 days with the cinnamic acids listed in Table 3 are glucose esters. The esters formed from *p*-coumaric, caffeic, ferulic and sinapic acids are identical in every way with material isolated from natural sources. During metabolism, glucose is attached to the carboxyl in preference to a phenolic hydroxyl group of the cinnamic acids in all the plants studied. Even if the carboxyl group is protected and a compound such as methyl *p*-coumarate is fed, the product is not the expected phenolic glucoside but the glucose ester (in this instance, 1-*p*-coumaroylglucose). This must be formed via the free cinnamic acid which was also detected after feeding and is presumably produced on hydrolysis of the methyl ester by one of the well-known plant esterases. In this study, the only plant found to produce phenolic glucosides is the tomato; caffeic acid is metabolized in the leaves mainly to caffeoylglucose but small amounts of the 3- and 4-$\beta$-glucoside of caffeic acid are also formed.

Plants allowed to metabolize *o*-coumaric acid for longer periods (3–7 days) produce the glucose ester accompanied by lesser amounts of the glucosylglucose (probably gentiobiose) ester. No appreciable quantities of the related quinic esters appear to be formed during the course of these experiments. Thus a careful search for *p*-coumaroylquinic acid among the metabolites of *p*-coumaric acid, in plants not naturally containing this ester, was completely unsuccessful. A similar examination of plants for chlorogenic acid after feeding caffeic acid

Table 3. *Sugar derivatives formed in leaves fed with hydroxycinnamic acids and related compounds*

Relative amounts formed are indicated thus: + + +, over 50%; + +, about 20%; +, 10% or less. Plant species are: A, various cultivated and wild *Solanum* species; B, *Cestrum newellii*; C, *Raphanus sativus*; D, *Clematis lawsoniana*; E, *Lycopersicum esculentum*; F, *Datura knightii*.

| Compound fed | Sugar derivatives produced | Plant species |
|---|---|---|
| L-Phenylalanine | 1-*p*-Coumaroylglucose | B |
| | 1-Feruloylglucose (+) and 1-*p*-coumaroylglucose (+ + +) | A |
| Cinnamic acid | 1-Cinnamoylglucose (+) and 1-*p*-coumaroylglucose (+ + +) | A, C |
| | 1-*p*-Coumaroylglucose | B, D, E |
| *o*-Coumaric acid | 1-*o*-Coumaroylglucose | A, D |
| | 1-*o*-Coumaroylglucose (+ + +) and 1-*o*-coumaroylgentiobiose (+ +) | E |
| *m*-Coumaric acid | 1-*m*-Coumaroylglucose (+), 1-feruloylglucose (+ + +) and caffeic acid 4-$\beta$-glucoside (+) | A, E |
| *p*-Coumaric acid | 1-*p*-Coumaroylglucose | A, D, E |
| | 1-Caffeoylglucose (+ +) and 1-*p*-coumaroylglucose (+ + +) | C |
| Caffeic acid | 1-Caffeoylglucose | A, B, D |
| | 1-Caffeoylglucose (+ + +), caffeic acid 3-$\beta$- (+) and 4-$\beta$-glucoside (+) | E |
| | Scopolin | F |
| Ferulic acid | 1-Feruloylglucose | A, E |
| Sinapic acid | 1-Sinapoylglucose | A, E |
| 3:4:5-Trimethoxycinnamic acid | 1-(3:4:5-Trimethoxycinnamoyl)glucose | A, E |

was not so easy, since this ester occurs naturally in large amounts in many plant species. However, no chlorogenic acid was found in the leaves of radish or of *Clematis lawsoniana* plants either before or after feeding with caffeic acid.

Several other products, besides the simple glucose esters, have also been found (Table 3). The major product of feeding cinnamic acid to potato and radish is not cinnamoylglucose, but 1-*p*-coumaroylglucose, presumably formed by *p*-hydroxylation *in vivo*. The introduction of a *m*-hydroxyl group into *p*-coumaric acid occurs in radish leaves; 1-caffeoylglucose is a minor metabolite of *p*-coumaric acid in this plant. *o*-Hydroxylation is also carried out in radish leaves, since caffeic acid is converted into a mixture of 1-caffeoylglucose and the related coumarin, aesculin. Similarly, scopolin is formed from caffeic acid in the leaves of *Datura knightii*. Hydroxylation and methylation are both involved in the unexpected production in some solanaceous plants of 1-feruloylglucose from *m*-coumaric acid. A number of cinnamic acid precursors were also fed to potato leaves. These were DL- and L-phenylalanine, L-tyrosine, phenylpyruvic acid, β-phenylpropionic acid and DL-2-phenyl-lactic acid. Of these, L-phenylalanine is the only effective precursor, large quantities of 1-*p*-coumaroylglucose, accompanied by traces of 1-feruloylglucose, being formed after feeding. Other solanaceous plants also convert phenylalanine into 1-*p*-coumaroylglucose; in contrast, leaves of the radish and *Impatiens balsamina* were not able to utilize phenylalanine in this way.

The results described above were obtained by feeding cinnamic acids through the petioles of leaves and also, for the potato and radish, by infiltration into tuber and root disks. Metabolic experiments were carried out mostly with leaves of the potato and other Solanaceae but a sufficient number of plants of other families was examined to show that glucose ester formation is a general property of most higher plants. It is also clear that plants vary in the ways they metabolize these cinnamic acids to other derivatives. In general, the nature of the cinnamic acid derivatives produced in the leaves of a particular plant appeared to be independent of the environment that plant was grown in, or the age of the leaves when sampled.

The possibility that some of the products of feeding experiments shown in Table 3 occur naturally in trace amounts cannot be ruled out. One such product, scopolin, was identified as a trace constituent of untreated *Datura knightii* leaves. However, spectral measurements on carefully purified samples from leaves fed with water or caffeic acid solution indicated that 8·2 times more scopolin was produced as a result of the feeding. There can thus be no doubt that caffeic acid is an efficient precursor of scopolin in this plant.

## DISCUSSION

Since the majority of natural phenols occur in plants as glycosides, it is not surprising to find that cinnamic acids also occur frequently in Nature in combination with sugars. The fact that simple glucose esters have not been described before may be attributed to the earlier emphasis on the quinic esters, especially on chlorogenic acid, which was isolated first by Payen (1846) and later characterized by Görter (1908). It is now apparent that, in a number of surveys, chlorogenic acid has not been satisfactorily distinguished from 1-caffeoyl- or 1-feruloyl-glucose. Indeed, 1-feruloylglucose and chlorogenic acid have identical $R_F$ values in a number of common solvent systems and can only be separated in butanol–ammonia or butanol–ethanol–water. The glucose esters of *Antirrhinum majus* were first thought to be quinic derivatives (Schütte & Böhme, 1958), partly because only quinic esters were known to occur widely at that time. Now glucose esters are known to occur widely, it seems probable in retrospect that the caffeic acid ester (designated as 'band 510'), isolated by Sondheimer (1957) from green coffee beans and several common fruits, was 1-caffeoylglucose.

Only three different sugars (glucose, gentiobiose and rutinose) have been found in cinnamic acid esters, but it is probable that others await recognition. Already, Kameswaramma & Seshadri (1947) have described a compound, pajaneelin, which, on hydrolysis, gives *p*-coumaric acid and fructose. Besides the simple glucose esters, there are a number of complex glucose derivatives of cinnamic acids, which are more difficult to characterize. A few such compounds have already been described and two substances found during the present investigation fall into this class. One of these (CA 5) is a caffeic acid derivative which differs from all the known caffeic acid–sugar compounds (see Table 4).

The discovery that glucose esters are formed from free hydroxycinnamic acids when fed to plants is unexpected, in view of two facts. First, that most $C_6$ phenols are rapidly converted into the corresponding β-glucosides when infiltrated into plant tissue (Hutchinson, Roy & Towers, 1958; Pridham & Saltmarsh, 1960); and, secondly, that Kosuge & Conn (1959) found that *o*-coumaric acid was converted into its phenolic glucoside (melilotoside) in sweet-clover leaves. A different enzyme from that required for synthesizing phenolic glucosides (Cardini & Yamaha, 1960) is presumably required for ester formation. Esterification

Table 4. *Naturally occurring caffeic acid glycosides and their $R_F$ values*

| Name | Sugars and other residues | $R_F$* in solvent *A* | $R_F$* in solvent *D* | Reference |
|---|---|---|---|---|
| 1-Caffeoylglucose | Glucose (1) | 0·51 | 0·63 | This paper |
| 1-Caffeoylgentiobiose | Glucose (2) | 0·29 | 0·47 | This paper |
| Caffeic acid 3-β-glucoside | Glucose (1) | 0·46 | 0·55 | This paper |
| Caffeic acid 4-β-glucoside† | Glucose (1) | 0·29 | 0·46 | Klosterman & Muggli (1959) |
| Echinacoside | Glucose (2), rhamnose (1), 3:4-dihydroxyphenylethanol (1) | 0·44 | 0·31 | Stoll, Renz & Brack (1950) |
| Compound CA 5 | Glucose (1) | 0·29 | 0·18 | This paper |
| Orobanchin‡ | Glucose (1), rhamnose (1) and catechol deriv. (?) | 0·56 | 0·63 | Bridel & Charaux (1925) |

* Chlorogenic acid has $R_F$ 0·59 in solvent *A*, $R_F$ 0·50 in solvent *D*.
† Only known to occur naturally in combined form, in the seed of flax, *Linum usitatissimum*.
‡ This compound, occurring in *Orobanche* plants, is incorrectly claimed to be chlorogenic acid by Privat (1959). Buddleoside, a compound present in *Buddleia davidii*, is either identical with, or very similar in structure to, orobanchin (W. D. Ollis, personal communication).

probably occurs in preference to glycosylation because it both neutralizes the acid grouping and also improves the sap solubility of the cinnamic acids.

Some of the results obtained have a bearing on the biosynthesis of cinnamic acids and related compounds. Levy & Zucker (1960) have suggested that the hydroxylation of cinnamic to *p*-coumaric to caffeic acid *in vivo* occurs with the quinic esters, and not with the free acids. The present results show that hydroxylation can equally well take place with the glucose esters as enzyme substrates. The role of L-phenylalanine and cinnamic acid as precursors in the synthesis of the hydroxycinnamic acids in *Salvia splendens* (McCalla & Neish, 1959) and of chlorogenic acid in potato disks (Levy & Zucker, 1960) has now been extended to cover the hydroxycinnamic acids of the leaves of potato, tomato and other plants. It thus appears that the route:

phenylalanine → cinnamic acid → *p*-coumaric acid

is a fairly general one for hydroxycinnamic acid biosynthesis.

It has often been suggested (see, for example, Grisebach & Ollis, 1961) that cinnamic acids can be converted *in vivo* by *o*-hydroxylation followed by *trans–cis* isomerism and intramolecular cyclization into the related coumarins. The present discovery that aesculin and scopolin are formed from caffeic acid in the radish and *Datura knightii* respectively shows that such a pathway is followed in some plant tissues.

Finally, it is apparent that cinnamic acid glucose esters are as important as the well-known quinic esters in the economy of plant cells. The ease with which they are formed from the free acids, their turnover in young radish leaves and their presence in many plants in amounts much smaller than the quinic esters suggest that they may be more important biosynthetic intermediates than compounds such as chlorogenic acid.

## SUMMARY

1. Fifteen hydroxycinnamic acid–sugar derivatives have been isolated from plants or produced by feeding plants with cinnamic acids.

2. They are the glucose esters of cinnamic, *o*-, *m*- and *p*-coumaric, caffeic, ferulic, sinapic and 3:4:5-trimethoxycinnamic acid, caffeoyl and *o*-coumaroyl-gentiobiose, *p*-coumaroylrutinose and caffeic acid 3- and 4-β-glucoside. The remaining two cinnamic acid derivatives contain glucose and different unidentified residues.

3. 1-*p*-Coumaroyl-, 1-caffeoyl- and 1-feruloyl-glucose occur widely in the leaves and flowers of many plants. In contrast, caffeic acid 3-glucoside is known only in the berries of wild potato species.

4. Free cinnamic acids, when fed to plants, are generally converted into the corresponding glucose esters. Caffeic acid is also converted in some plants into aesculin or scopolin or into a mixture of its 3- and 4-β-glucoside. The hydroxylation and methylation of cinnamic acids has been observed *in vivo*.

5. It is suggested that the simple hydroxycinnamic acid glucose esters may be important biosynthetic intermediates in plants.

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# The Synthesis of *meso*Inositol in Germ-free Rats and Mice

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The precise role of dietary *meso*inositol in mammalian nutrition remains controversial despite more than two decades of inquiry. Early reports linking inositol to the promotion of growth and prevention of alopecia in mice (e.g. Woolley, 1940, 1941) and rats (e.g. Pavcek & Baum, 1941; Cunha, Kirkwood, Phillips & Bohstedt, 1943) have not been uniformly reduplicated (e.g. Martin, 1941; Fenton, Cowgill, Stone & Justice, 1950; Ershoff & McWilliams, 1943; McCormick, Harris & Anderson, 1954). In addition, no characteristic syndrome of inositol deficiency has yet been described in man. However, the recent findings that added inositol is required for the propagation of human cells in tissue culture (Eagle, Oyama, Levy & Freeman, 1957), and unbound inositol is concentrated in most mammalian tissues (Dawson & Freinkel, 1961) prompted a re-examination of the endogenous biosynthesis of inositol.

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Excellent relevant data were available in this area before the inception of the present studies. Needham's (1924) observations of sustained inositoluria in rats maintained on low inositol diets had been re-enforced by the independent reports of Daughaday, Larner & Hartnett (1955) and Halliday & Anderson (1955) that [$^{14}$C]inositol could be recovered from rats given repeated injections of [$^{14}$C]glucose. However, as was first suggested by Woolley (1942), such 'endogenous' inositol could have originated from alimentary tract micro-organisms which are known to profoundly affect the picture in other fields of mammalian metabolism, e.g. the [$^{14}$C]urea catabolism of mammals is entirely due to associated micro-organisms (Kornberg, Davies & Wood, 1954). To minimize intestinal contributions, Daughaday *et al.* (1955) excised the entire gut immediately before analysis of the carcass for inositol. The possibility of microbial biosynthesis and absorption of labelled inositol from the intestine during the 3 days

preceding killing was not thereby excluded. Similarly, although Halliday & Anderson (1955) effected prolonged reduction of intestinal flora by feeding antibiotics and caecectomy, the potential for inositol synthesis by the residual intestinal yeasts was not eliminated. An alternative approach was employed by Luckey, Pleasants, Wagner, Gordon & Reyniers (1955), who maintained complete intestinal sterility in two germ-free rats during chronic balance studies. However, owing to 'the variation in inositol analyses of different lots of diet', they were unable to interpret their recovery of 74 and 40 % more inositol than had been fed as definite evidence for net biosynthesis.

For the present experiments, the isotopic and germ-free techniques were combined. [1-$^{14}$C]-Glucose was acutely administered to germ-free mice and rats and recovery of [$^{14}$C]inositol from the carcass was contrasted with values obtained under identical conditions in normal animals.

## EXPERIMENTAL

*Animals.* Five days before the studies with radioactive glucose on 26 October 1959, germ-free rats (born 21 September 1959), germ-free mice (born 13 September 1959) and normal rats and mice (born 10 September 1959) were sent by air-express from the Lobund Institute, Notre Dame, Ind., U.S.A., to the Charles River Laboratories, Dover, Mass., U.S.A. A germ-free isolator (Reyniers, 1959) containing food, water, cages, dissection tools and cotton swabs was employed for the transport and subsequent maintenance of the germ-free animals. At the end of the [$^{14}$C]glucose experiments, swabbings were obtained from the inside walls of the isolator and from the surfaces and freshly expressed faeces of the germ-free animals. Sterile cultures from these swabbings in thioglycollate broth and on blood–agar plates indicated that contamination had not occurred during transport and maintenance of the animals at the Charles River Laboratories. All animals were generously donated by Professor P. C. Trexler of the Lobund Institute; maintenance of isolators and assistance with germ-free technique were generously provided by Dr H. L. Foster and his associates of the Charles River Laboratories.

*Injection solutions.* Chromatographically pure [1-$^{14}$C]-glucose (Nuclear-Chicago Corp., Ill.) was combined with unlabelled glucose (National Bureau of Standards) to prepare an injection solution containing 9·8 mg. of glucose and 30 $\mu$c of $^{14}$C/ml. The material was sterilized by autoclaving for 20 min. with 16 lb./in.$^2$ pressure. An ampouled portion together with appropriate syringes and needles was introduced into the isolator by passage through the supply lock, by using cold sterilization with a peracetic acid spray.

To establish that the [1-$^{14}$C]glucose was not contaminated with radioactive inositol, any contamination was concentrated by growing the inositol-dependent yeast *Kloeckera brevis* in the presence of the [$^{14}$C]glucose. A sample (1 ml.) of the glucose injection solution was added to the growth medium of Campling & Nixon (1954). Carrier inositol (4·8 $\mu$g.) was added and the mixture inoculated with *K. brevis*. The yield of *K. brevis* cells obtained after 3 days' growth at 25° was normal as judged by turbidity readings (4·9 $\mu$g.). The cells, which previous experiments had shown contain all the inositol in combined form (R. M. C. Dawson & N. Freinkel, unpublished work), were hydrolysed with 6 N-HCl for 40 hr. at 105°. To the hydrolysate was added 50 $\mu$g. each of inositol and glucose as carriers and it was then dried *in vacuo*. The residue was dissolved in water and passed through a column of mixed-bed ion-exchange resin, Amberlite MB-1 (8 cm. long × 0·8 cm. diameter). The effluent and washings were evaporated to low volume and applied as spots to two paper-strip chromatograms 2·5 cm. wide. These were developed with the solvents and conditions described below, and, after drying, passed through an automatic micromil-window strip scanner (Nuclear-Chicago Actigraph). The strips were treated with acetone–$AgNO_3$ and ethanolic NaOH reagents to detect the inositol and glucose spots (Trevelyan, Procter & Harrison, 1950). All of the radioactivity was confined to the glucose area. Under the conditions of the test 0·001 % of the radioactivity occurring as *meso*inositol would readily have been detected.

## METHODS

*Experimental work on germ-free and normal animals.* All studies were performed with male paired germ-free and normal animals. Samples of the glucose injection solution were administered at hourly intervals intraperitoneally as follows: one germ-free and one control rat were each given three 1 ml. injections; one germ-free and one control rat each received six 0·5 ml. injections; and one germ-free and one control mouse were each treated with three 0·5 ml. injections. The germ-free animals were injected by using the rubber manipulating gloves in the isolator. All animals were allowed free access to food throughout the experimental period. The animals were killed by a blow on the head 1 hr. after the last injection of each pair. Immediately thereafter, the animals were homogenized with 11 N-$H_2SO_4$. Mice were introduced directly into 50 ml. of 11 N-$H_2SO_4$ contained in a Waring Blendor. Separate blenders were employed for each animal. Rats were preliminarily sectioned into smaller pieces and ground with a meat grinder into 100 ml. of 11 N-$H_2SO_4$/100 g. body wt. contained in Waring Blendors. Subsequent processing was by a modification of the method of Halliday & Anderson (1955).

The suspensions (animals homogenized with 11 N-$H_2SO_4$) were hydrolysed in sealed bottles at 16 lb./in.$^2$ pressure for 12·3 hr. at 122°. The black hydrolysates were cooled and to each was added 25 mg. of carrier inositol. The hydrolysates were diluted with an equal volume of water and solid $CaCO_3$ was added in small portions until they were at about pH 5. The precipitate of $CaSO_4$ and other insoluble matter was filtered off under pressure and the precipitate washed well with water. The filtrate and washings were combined and decolorized with Darco G 60 charcoal. This was most effectively carried out in the cold with successive small portions of charcoal, and filtering before using the next portion. Samples of the clear filtrate were retained for inositol assay (Campling & Nixon, 1954) as described by Freinkel, Dawson, Ingbar & White (1959). The remainder of the filtrates were passed through columns of mixed-bed ion-exchange resin, Amberlite MB-1 (2 in. diameter, 12 in. long for mouse experiments, 14 in. long for germ-free rat filtrates, and 17 in. long for control rats). The effluents and

washings were free of $SO^{2-}$ ions when tested with $Ba^{2+}$ ions. They were evaporated to low volume (about 0·5 ml.) and 4 ml. of ethanol was added to induce crystallization. The separated crystals were dissolved in 2 ml. of water. The mixture was centrifuged, and the supernatant separated from debris, evaporated to low volume (0·25–0·5 ml.) and treated with 2–3 ml. of ethanol. After the inositol had been allowed to crystallize for 1 hr. at 0°, it was collected and recrystallized from aqueous ethanol. The crystals were dried (e.g. for animal no. 6: melting point, 222·5° uncorr.). The samples of crystalline inositol were dissolved in 5 ml. of water. Samples (1 ml.) were evaporated on planchets for radioactive assay with an automatic micromil-window flow counter (Nuclear-Chicago Corp.) and a further sample (0·1 ml.) was used for microbiological inositol assay (Campling & Nixon, 1954).

*Chromatography of inositol samples.* Samples of the above inositol solutions equivalent to 1·2 mg. were run on paper chromatogram strips 5 in. wide. Two solvent systems were used: (1) propanol–ethyl acetate–water (24:13:7, by vol.) and (2) propan-2-ol–acetic acid–water (3:1:1, by vol.), both of which give a good separation of inositol from sugars such as glucose. After the chromatograms had been run (40 hr., descending) they were dried and a central strip $\frac{1}{4}$ in. wide was cut out and treated with the acetone–$AgNO_3$ and ethanolic NaOH reagents for detecting polyols (Trevelyan *et al.* 1950). Only an inositol spot was rendered visible, and the strips on either side of this were cut out of the untreated paper and extracted twice over 10 hr., each time with 20 ml. of water. A sample (0·05 ml.) was taken for inositol assay and the remainder (0·95 ml.) transferred to a planchet for counting.

For radioactive assay, sufficient counts were observed to reduce the probable error of the measurement to less than 3%. All planchets were appropriately corrected for self-absorption to a mass of 5 mg.

## RESULTS

Results are summarized in Table 1. In the rats, total recovered inositol, after correction for added inositol carrier, averaged 27·7 mg./100 g. body wt. in the two germ-free, and 30·1 mg./100 g. body wt. in the two normal, animals. The findings are in substantial agreement with the average values for carcass inositol of 28·1 mg./100 g. which Halliday & Anderson (1955) obtained in three normal rats by a similar fractionation procedure, and slightly greater than the 17·3 mg./100 g. (Daughaday *et al.* 1955) and 23·3 mg./100 g. (McCormick *et al.* 1954) recovered by somewhat different techniques. Incomplete recovery of inositol precluded estimation of total carcass inositol in the germ-free mouse. The value of 27·8 mg./100 g. in the control mouse is somewhat less than the average of 39·2 mg./100 g., which Woolley (1942) observed in six mice fed on a stock diet for 4 weeks after weaning.

Significant quantities of radioactivity were present in the inositol crystallized from the carcasses of all mice and rats after the administration of [1-$^{14}$C]glucose. That this constituted newly synthesized [$^{14}$C]inositol was established for each animal by the demonstration of constant inositol specific radioactivity during chromatographic resolution in two solvent systems. In this determination of specific radioactivity the microbiological assay of inositol gave values within about ±6% and the radioactivity was determined within ±3%. Further, the [$^{14}$C]inositol isolated from one animal was used to support the growth of *K. brevis*. The yeast was then recovered from the culture medium, it was hydrolysed with acid and inositol was then isolated from the hydrolysate by strip chromatography. The specific radioactivity of the isolated and original inositol were identical (315 and 314 counts/min./mg. of inositol), confirming that the $^{14}$C had been incorporated into *meso*inositol. Similar experiments reported in the Experimental section had already shown that the [$^{14}$C]glucose injected was free of *meso*[$^{14}$C]inositol.

In the paired rats and mice killed 3 hr. after the first injection of [1-$^{14}$C]glucose, the total counts

Table 1. *The formation of radioactive inositol from* [1-$^{14}$C]*glucose* in vivo

| Animal | Weight (g.) | Duration of experiment (hr.)* | Recovered inositol (mg.)† | Recovery of [$^{14}$C]inositol: Counts/mg. of inositol/min.‡ *A* | *B* | Average | Total counts | Per cent of [1-$^{14}$C]glucose administered |
|---|---|---|---|---|---|---|---|---|
| Rats | | | | | | | | |
| Germ-free | 104 | 3 | 51·5 | 138 | 126 | 132 | 6 798 | 0·0279 |
| Control | 180 | 3 | 75·5 | 131 | 134 | 132·5 | 10 004 | 0·0410 |
| Germ-free | 108 | 6 | 57·3 | 315 | 355 | 335 | 19 195 | 0·0787 |
| Control | 180 | 6 | 83·0 | 172 | 156 | 164 | 13 612 | 0·0558 |
| Mice | | | | | | | | |
| Germ-free | 26 | 3 | 22·5 | 259 | 259 | 259 | 5 827 | 0·0478 |
| Control | 36 | 3 | 35·0 | 232 | 259 | 245·5 | 8 592 | 0·0704 |

* Interval elapsing between the first of a series of injections of [1-$^{14}$C]glucose and killing. For details of administration schedules, see text.

† Before fractionation 25·0 mg. of carrier inositol was added to each carcass.

‡ Specific radioactivities of inositol (counts/min./mg.) isolated after paper chromatography of crystalline material in: (*A*) propanol–ethyl acetate–water, and (*B*) propan-2-ol–acetic acid–water. Average chromatographic specific activities were employed to derive minimal estimates of 'total counts' recovered as radioactive inositol from each animal.

recovered as radioactive inositol were greater in the control than in the germ-free animals. In the paired rats killed at 6 hr., total recovered radioactive inositol was greater in the germ-free animal. Paired values did not differ by more than 50 % in any instance, thus suggesting that intestinal microflora do not appreciably influence the fractional rates of endogenous inositol biosynthesis from administered [$^{14}$C]glucose in these species.

## DISCUSSION

In the present experiments it has been established that the [$^{14}$C]glucose injected contains no contaminating *meso*[$^{14}$C]inositol. On the other hand, the crystalline *meso*inositol of the correct melting point isolated from the carcasses of both germ-free and normal animals after [$^{14}$C]glucose injection contained appreciable radioactivity. That this did not represent contamination of the isolated inositol with another metabolic product formed from the [$^{14}$C]glucose relies on the findings: (*a*) that the specific activity of the [$^{14}$C]inositol remained constant on paper chromatography in two solvent systems; (*b*) that it remained constant after a growth cycle through *K. brevis* and reisolation of the inositol from the yeast.

These results leave little doubt that normal and germ-free animals can biosynthesize labelled inositol, after the injection of [1-$^{14}$C]glucose. Since the completion of these studies, Eagle, Agranoff & Snell (1960) have reported the occurrence of definite, albeit limited, synthesis of inositol from radioactive glucose in isolated human tissue culture. The combined observations would indicate that mammalian tissues are capable of inositol production even without microbial synergy, and that all of the mammalian requirements for inositol need not be met exogenously. It cannot, however, be assumed without qualification that the unknown metabolic processes which synthesize [$^{14}$C]inositol result in a net formation of inositol which is available for the animal economy, for it is conceivable that if such processes are reversible, catabolism of inositol will outstrip synthesis.

Kinetics of glucose metabolism have been sufficiently documented in the rat (Feller, Strisower & Chaikoff, 1950) to justify efforts at more quantitative treatment of the present results. In control rats, 0·041 and 0·056 % of the counts in the administered [1-$^{14}$C]glucose could be recovered as radioactive inositol after experimental periods of 3 and 6 hr. respectively. The figures are in the same neighbourhood as the values of 0·05 and 0·07 % obtained by Halliday & Anderson (1955) when normal rats were killed 2 hr. after the completion of a series of ten injections of [1-$^{14}$C]glucose at 1 hr. intervals. It seems reasonable to assume therefore that 0·05 % represents an approximate minimal figure for the conversion at equilibrium. Since the half-time for the turnover of glucose in the rat is less than 1 hr. (Feller *et al.* 1950), and since not enough glucose was injected at any time to disrupt equilibrium conditions, it might be anticipated that most of the labelled glucose in all of the studies had equilibrated with the exchangeable glucose pool. In the rat, turnover of body glucose amounts to 100 mg./hr./100 g. body wt. (Feller *et al.* 1950). Thus, taking 0·05 % as a minimal figure for the conversion at equilibration, this may be extrapolated for 24 hr. to estimate that a minimum of about 1·2 mg. of glucose/100 g. of rat is converted into inositol per day. Moreover, unless the metabolism and distribution of glucose are altered by intestinal sterility, the fact that fractional conversions of [1-$^{14}$C]glucose were of similar magnitude in the germ-free rats (i.e. 0·03 and 0·08 % at 3 and 6 hr. respectively) would suggest that the minimal estimate of 1·2 mg. may equally obtain in these animals. Unfortunately, translation of the values for glucose into actual quantities of inositol is precluded by the lack of information about pathways of inositol biosynthesis. If conversion of glucose into inositol were equimolar, then the results would be compatible with a minimum synthesis from glucose of 1·2 mg. of inositol/100 g. of rat/day. However, at least in yeast, such an equimolar transformation does not obtain (Charalampous, 1957), and consequently in this species the cyclization theory of inositol formation from glucose (Fischer, 1945) does not appear to be tenable. Thus, although the present studies constitute clear evidence of endogenous inositol biosynthesis, they do not resolve the question of the nutritional adequacy of this process.

## SUMMARY

1. The biosynthesis of inositol from glucose has been examined in normal and germ-free rats and mice.

2. After the acute intraperitoneal administration of [1-$^{14}$C]glucose, [$^{14}$C]inositol could be isolated from all animals. In paired germ-free and control rats and mice, the recoveries of radioactive inositol were of the same order of magnitude.

3. The findings would indicate that inositol can be formed from glucose in these species and that endogenous biosynthesis does not require the presence of alimentary canal micro-organisms.

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# Changes in Microsomal Components Accompanying Cell Differentiation of Pea-Seedling Roots


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This paper describes some changes found in the microsomes of pea roots during growth (see Whaley, Mollenhauer & Leech, 1960, for a review on plant microsomes). The growing-plant root provides a convenient source of material, because growth is largely in one direction and cell division is confined to the apex, so that it is possible to obtain batches of cells in progressive stages of development by cutting serial transverse segments. Small segments of the root tips were used, so that differences between the meristem and the first stages of differentiation could be studied. The experiments depend on the chance observation that the sedimented microsomal pellet may be readily separated into two components, one particulate and the other largely membranous. These components were examined by their protein and nucleic acid contents, by electrophoresis and by electron microscopy.

A preliminary communication of these results has been published (Loening, 1960).

## MATERIALS AND METHODS

*Preparation of root-tip segments.* Pea seeds of the variety Meteor (Sutton and Sons, Reading) were sown in horticultural vermiculite–soft tap water (3:1, v/v) and germinated in the dark at 25° for 48 hr. The roots were then 3–4 cm. long; exceptionally long or short ones were rejected. The roots were cut into three serial segments, the first tip segment being 1·6 mm. long, the second 1·8 mm. and the third basal segment 3·0 mm. To facilitate the cutting of large numbers of tips, a block of four Perspex sheets of thicknesses 1·6, 1·8, 3·0 and 6·0 mm. was drilled with 55 holes of suitable diameter to hold the roots. The block was placed, thinnest sheet downwards, flat on a sheet of glass and the roots were inserted into the holes so that the tips touched the glass. The protruding older parts of the roots were sliced off flush with the Perspex with a microtome blade and the block was inverted. The Perspex sheets were then removed one by one and the exposed segments sliced off. The whole operation took 10–15 min.

The lengths of the segments are chosen so that the first includes the meristem (potentially dividing cells) and the root cap, but few developed or elongating cells. The segment consists therefore of largely undifferentiated tissue. The second segment is a zone of increased metabolic activity, including protein synthesis (see Heyes & Brown, 1956). There is some cell enlargement and cell vacuoles appear. This segment therefore shows the beginnings of differentiation. The oldest part of the second segment and the youngest of the third is the zone with the most rapid rate of cell elongation. Cells of the third segment are very much larger and vacuolated, although the final cell volume is not reached until some 10–11 mm. from the tip. The average weight and number of cells per segment is shown in Table 1.

Table 1. *Weights and numbers of cells of root segments*

Serial segments were cut as described in the text. Cell counts are the averages of six determinations of a batch of 20 segments, by the method of Brown & Broadbent (1950).

| Segment | Length (mm.) | Wt. (mg.) | $10^{-4}$ × No. of cells | $10^2$ × Average wt./cell ($\mu$g.) |
|---|---|---|---|---|
| 1 | 1·6 | 0·837 | 10·6 | 0·79 |
| 2 | 1·8 | 1·61 | 8·97 | 1·8 |
| 3 | 3·0 | 3·07 | 6·12 | 5·0 |

*Preparation of microsomes.* The root segments were cooled as soon as cut, to 0–5°, and all subsequent manipulations were done at this temperature. The segments were ground by hand in a glass homogenizer, 165 segments in 3 ml. of a medium at pH 6·9, containing 4 mM-$KH_2PO_4$, 2·5 mM-tris, 0·01 mM-$MgSO_4$ and 0·5 M-sucrose, being used. The buffer concentration was low since it was found that high ionic concentrations dissolved some microsomal protein and RNA. The $Mg^{2+}$ ion concentration was that used by Webster (1957) for amino acid-incorporation experiments in pea-seedling microsomes. In a few experiments a similar medium at pH 7·5 containing 4 mM-tris (instead of 2·5 mM) was used, and in others 0·4 M-sucrose alone.

The homogenate was centrifuged at 12 500*g* for 30 min. to sediment cell debris, nuclei and mitochondria. The supernatant was centrifuged at 30 000*g* for 30 min. Very little material was sedimented under these conditions, indicating that the sedimentation properties of the mitochondria and microsomes are different and that few intermediate-sized particles are present. The small sediment obtained will be referred to as fraction X and is discussed in more detail below. The remaining supernatant contained a little white floating material and the microsomes had begun to sediment a little, as indicated by schlieren effects on stirring. The whole supernatant was decanted and mixed, and portions (1 ml.) were pipetted into 7 ml. Lustgroid Spinco ultracentrifuge tubes. The tubes were filled with *n*-heptane and centrifuged in the 40·2 rotor at 40 000 rev./min. for 90 min. This gives an average centrifugal force in the 1 ml. sample of about 140 000*g*. The microsomal pellets obtained were almost transparent, colourless or pale yellow from the first segment and yellow from segments 2 and 3. The tubes were inverted to drain and as much as possible of the supernatant liquid was removed with filter paper.

*Separation into two microsomal components.* If the tubes were left inverted for 5–15 min., the yellow part of each pellet slid down the side of the tube, leaving behind a small colourless firm pellet. Two microsomal components were thus obtained. The yellow sliding pellet will be referred to as 'light' microsomes and the colourless firm one as 'heavy' microsomes. Preparations from the first root segment gave no 'light' microsomes which could be distinguished from traces of the supernatant liquid. 'Light' microsomal pellets from the second root segment were slightly gelatinous and tended to spread out while sliding down the tubes. Those from the third segment retained their circular shape and were so gelatinous that the pellets could be picked up intact with a spatula. The separation of the 'light' and 'heavy' components was hastened, and the 'light' microsomes were collected, by centrifuging the inverted tubes in larger glass tubes at up to 250*g* for about 5 min. The heavy microsomes remained as firm pellets in the Lustroid tubes, although there was a slight streak down the sides of the tubes, most in preparations from the first segment and least from the third. This pellet was similar to the pellets of deoxycholate-treated ribosomes obtained from animal or bacterial cells in physical consistency, transparency and lack of colour.

*Determination of ribonucleic acid and protein.* Each microsomal pellet was suspended in 1 ml. of 0·5 M-perchloric acid at 0° and the white precipitate was collected by centrifuging at 3000*g* for 10 min. The pellets were extracted with 1 ml. of perchloric acid for 20 min. at 70° and again centrifuged (Littlefield, Keller, Gross & Zamecnik, 1955). The supernatants were removed and diluted with 5 ml. of water. The nucleic acid contents of the extracts were measured spectrophotometrically at 260 m$\mu$ in a 1 cm. cell, assuming that 31 $\mu$g. of the hydrolysed RNA/ml. gives an extinction of 1·0. This compares with the value used by Ts'o & Sato (1959). The first cold acid extract gave readings about 2% of the total and was neglected. To confirm the value of the extinction coefficient RNA was also determined on a few samples by the orcinol method with correction for sucrose (Slater, 1958), yeast RNA being used as a standard, and by the difference in total nitrogen determinations before and after extraction with hot perchloric acid. The three methods agreed within experimental error except that the extinction at 260 m$\mu$ of the 'heavy' microsome samples indicated values 10–15% higher than those obtained by the other methods.

Protein was determined by the Kjeldahl method with 10% selenium in sulphuric acid as the digestion catalyst (Fawcett, 1954). The ammonia was determined by the Conway method in 3 cm. diameter diffusion dishes (Conway, 1957).

*Electrophoresis.* The cellulose acetate-membrane method devised by Kohn (1958) was used. A buffer at pH 6·7, containing 7·5 mM-citric acid and 35 mM-$K_2HPO_4$, was found to be the most satisfactory of several tried. A current of 2 mA/5 cm. width of paper (about 100 v/10 cm. length) was applied for up to 3 hr. The paper was then dried at room temperature. RNA was detected by contact printing on to Ilford Document paper 50 with a Hanovia u.v. lamp emitting at 254 m$\mu$. Protein was then stained on the same sheet, up to 0·005% of water-soluble nigrosine (Edward Gurr Ltd., London) in 5% trichloroacetic acid being used. In order to render the microsomal components soluble for electrophoresis, a number of techniques with detergents or ribonuclease were tried. The most satisfactory separations were obtained by suspending the microsomal pellets in about 5 vol. of the electrophoresis buffer containing 0·01% of pancreatic ribonuclease (Sigma Chemical Co.). Digestion of the clear suspension was continued at room temperature for 30–60 min., during which a precipitate appeared. Portions of the suspension were then applied to the acetate membrane.

*Electron microscopy.* The pellets were fixed in the homogenizing medium containing 1% of osmic acid, for 2–5 hr. at 0°. It was essential not to disrupt the pellets during fixation, otherwise they were easily lost, particularly the 'heavy' microsomes, which did not stain brown or black as much as did the 'light' ones. A pellet of 'heavy' microsomes was recovered by centrifuging the supernatant after

Table 2. *Yields of protein and ribonucleic acid in the cell fractions*

Batches of the three root segments were homogenized and centrifuged as described in the text. 'Debris' refers to all material, including mitochondria, sedimented at 12 500*g* for 30 min.; fraction X, 30 000*g*, and microsomes, 140 000*g*, for 90 min. Figures are the averages of two determinations.

| | Protein (percentage of total) | | | | Total protein (mg./g. fresh wt.) |
|---|---|---|---|---|---|
| Segment | 'Debris' | Fraction X | Microsome | Soluble | |
| 1 | 32 | 5·0 | 15 | 48 | 42 |
| 2 | 30 | 5·4 | 19 | 46 | 37 |
| 3 | 35 | 6·0 | 13 | 45 | 16 |
| | Ribonucleic acid (percentage of total) | | | | Total nucleic acid (mg./g. fresh wt.) |
| Segment | 'Debris'* | Fraction X | Microsome | Soluble | |
| 1 | 24 | 8·5 | 52 | 15 | 13 |
| 2 | 19 | 8·3 | 54 | 18 | 12 |
| 3 | 22 | 10·6 | 37 | 30 | 4·6 |

* Includes DNA.

fixation. The pellets were dehydrated with increasing concentrations of ethanol and left overnight in 1 % of phosphotungstic acid in ethanol (Wakid, 1960). After washing in ethanol they were embedded in methacrylate at 45°, sectioned and examined in the Siemens Elmiscop I at 40 kv and an objective aperture of 30 m$\mu$.

## RESULTS

*Yields of the cell components.* Table 2 shows the percentage distribution of the cell fractions obtained from the three root segments as described above. A large proportion of the RNA of the cell is sedimented with the microsomes, but the percentage falls slightly in the third segment. The yield of fraction X is small, particularly in terms of RNA, and increases slightly in the third segment. This fraction, however, can interfere with the separation of the 'light' and 'heavy' microsome components if it is not removed by centrifuging at 30 000*g*. It causes the 'heavy' pellet to break up when the inverted Lustroid tube is centrifuged at 250*g*, resulting in an apparently increased yield of 'light' microsomes and considerable streaking down the sides of the tubes. Thus although it is possible that fraction X includes some microsomal material, it behaves in a distinct manner during these isolation procedures, and on the basis of total yield it can only include a small proportion of the microsomes. It is believed therefore that the homogenization and centrifuging procedures described are satisfactory for the isolation of the bulk of the microsomes. The exact conditions have been found not to be critical.

*Yields of 'light' and 'heavy' microsomes.* The fact that the separation of the microsomal pellet into two components occurs in the inverted Lustroid tube under gravity alone, and yet the 'heavy' pellet remains intact at 250*g*, indicates that the two pellets have widely different physical properties. This is confirmed by the electron microscopy described below, in which it is shown that the 'heavy' microsomes consist of ribonucleoprotein particles and the 'light' of a mixture including vesicles. The separation was found to be reproducible and not to be sensitive to small changes in conditions. The amounts of 'heavy' and 'light' microsomes obtained from the three segments were determined in terms of their RNA and protein contents. The results are shown as $\mu$g. of RNA or of protein/cell in Table 3. The amount of 'heavy' microsomal protein remains constant at first and falls in the more mature cells of the third root segment, whereas the 'light' increases rapidly with

Table 3. *Yields of 'heavy' and of 'light' microsomes from the three root segments*

Yields are expressed as protein or RNA per cell in the three root segments. Averages of two determinations from one homogenization are given.

| | $10^5 \times$ Protein ($\mu$g./cell) | | $10^5 \times$ RNA ($\mu$g./cell) | |
|---|---|---|---|---|
| Segment | 'Heavy' | 'Light' | 'Heavy' | 'Light' |
| 1 | 4·72 | 0·92 | 5·14 | 0·78 |
| 2 | 4·85 | 8·99 | 7·45 | 5·28 |
| 3 | 2·48 | 9·49 | 4·26 | 5·12 |

Table 4. *Ratios of yields of 'light' to 'heavy' microsomes obtained by homogenization in different media*

| | Protein ratio of 'light':'heavy' microsomes | | RNA ratio of 'light':'heavy' microsomes | |
|---|---|---|---|---|
| Segment | pH 6·9 medium | Sucrose alone | pH 6·9 medium | Sucrose alone |
| 1 | 0·19 | 0·28 | 0·15 | 0·16 |
| 2 | 1·8 | 1·3 | 0·70 | 0·79 |
| 3 | 3·8 | 4·2 | 1·2 | 1·6 |

cell age. Most of this increase normally occurs between the first and second segments and there is little further change in the third. Occasionally an increase between the second and third segments was found, but then the yield of 'light' microsomal protein from the second segment was correspondingly low. These variations are probably caused by slight differences in the conditions of growth, resulting in different lengths of meristem. The results expressed in terms of RNA are similar except for the high RNA content of 'heavy' microsomes from the second segment.

Resuspension and re-centrifuging of the 'light' pellets gave a negligible further amount of 'heavy' and re-centrifuging of the 'heavy' gave no further 'light'. This shows that the separation obtained by this method was complete.

These results are unaffected by changes in the composition of the homogenization medium. A medium containing 1 mM-$Mg^{2+}$ ion and 0·5 mM-$Ca^{2+}$ ion, which would be expected to cause some aggregation of the particles, retarded the separation of the 'light' from the 'heavy' pellet but did not affect the results. Resuspension of the pellets in this medium caused appreciable precipitation of the microsomes. Sucrose alone was used as homogenizing medium by Ts'o, Bonner & Vinograd (1956) and Ts'o & Sato (1959), who isolated microsomes consisting entirely of ribonucleoprotein particles. Table 4 shows the ratios of 'light' to 'heavy' microsomes obtained by homogenization in the pH 6·9 medium and in sucrose alone.

Although small amounts of salts did not seem to affect the isolation of the microsomes, it was possible that material released from the cell vacuoles during homogenization could do so. It is for this reason, as well as convenience in handling small quantities, that a large volume of medium was used for homogenization. As a further check that different materials released by the three segments do not influence the yield of microsomes, the first segment was homogenized mixed with either the second or the third segment. The yields obtained were compared with those calculated from the sum of the tip and basal segments homogenized separately. The results are shown in Table 5. The agreement with the calculated values is good, considering the large differences between the three segments (see Table 4).

It is concluded therefore that the change in the relative amounts of the 'light' and 'heavy' microsomes with the age of the cells is not an artifact due to the homogenizing medium or to the different substances released by the three segments.

*Nucleic acid content of the microsomes.* Table 6 shows the ratio of RNA to protein in the 'light' and 'heavy' components. The values obtained for the 'heavy' component compare with the highest

Table 5. *Ratio of amounts of 'light' to 'heavy' microsomal protein and RNA obtained from the tip and basal segments mixed, compared with ratios calculated from the sum of segments homogenized separately*

Microsomes were isolated as described in the text: 82 3·4 mm. root tips (segments 1 and 2 together), or 82 each of segments 1 and 3 mixed, per 3 ml. of medium were used. Averages of two determinations are given.

| Segments | Yield measured as | Ratio obtained from mixed segments | Ratio calculated from sum of segments (from Table 3) |
|---|---|---|---|
| 1 and 2 | Protein | 0·87 | 0·98 |
| | RNA | 0·37 | 0·45 |
| 1 and 3 | Protein | 1·4 | 1·1 |
| | RNA | 0·43 | 0·49 |

Table 6. *Ratio of ribonucleic acid to protein of the microsome components*

Details are as given for Table 4.

| Segment | 'Heavy' microsomes | | 'Light' microsomes | |
|---|---|---|---|---|
| | pH 6·9 medium | Sucrose alone | pH 6·9 medium | Sucrose alone |
| 1 | 1·09 | 0·82 | 0·85 | 0·48 |
| 2 | 1·53 | 1·19 | 0·59 | 0·71 |
| 3 | 1·71 | 1·33 | 0·54 | 0·50 |

reported by Peterman, Hamilton, Balis, Samarth & Pecora (1958) for deoxycholate-treated animal microsomes. Peterman *et al.* reported a probable error in the values and there is likely to be a similar one for the 'heavy' microsomes, as mentioned in the Materials and Methods section. The values are nevertheless considerably higher than those obtained previously for plant tissues. The proportion of RNA increases with the age of the cells in 'heavy' microsomes but decreases in the third segment in 'light'. The net result is a decrease, since the total amount of the 'light' microsomes increases. The differences between the values obtained with the pH 6·9 medium or sucrose alone are probably not significant. The errors become large when the total yield is small, as in 'light' microsomes from the first segment, which were obtained in only just detectable quantities.

*Electrophoresis.* Fig. 1 shows the electrophoretic patterns obtained from the two microsome components after treatment with ribonuclease. The bands of the nucleic acid components were reproducible whereas the protein patterns varied slightly in different experiments. The 'light' microsomes show two positively charged bands, of which only traces appear in the 'heavy', in addition to some insoluble material. The protein pattern also shows

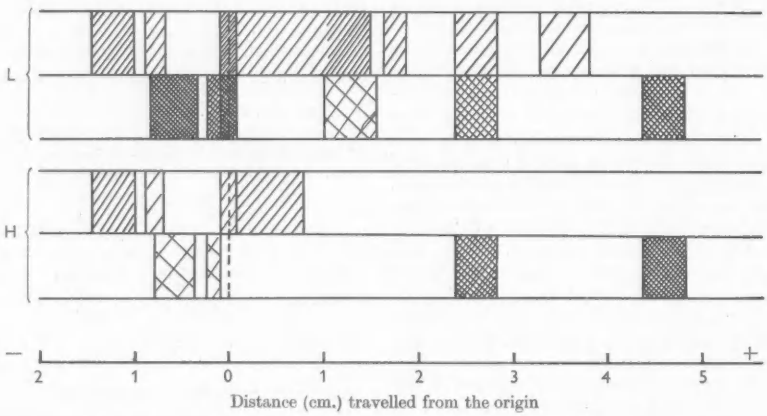



Fig. 1. Electrophoresis of 'light' and 'heavy' microsomes after treatment with ribonuclease. Diagonal shading represents protein fragments, and the cross-hatching, ribonucleic acid fragments. Density of shading represents the approximate strength of the staining or u.v. absorption, the lightest being barely detectable. L, 'Light' microsomes; H, 'heavy' microsomes. Patterns obtained from the three root segments were identical except that the yield of 'light' microsomes from the first segment was too small to use. The most cathodic protein band is the ribonuclease.

a greater complexity in the 'light' than in the 'heavy' microsomes. Except for the insoluble material there is little association between nucleic acid and protein.

These electrophoretic separations are sufficient to show that the two microsome fractions are in part different, the 'light' being more complex. No differences, however, could be detected between preparations from the different root segments.

*Electron microscopy.* Plate 1 shows (A) an electron micrograph of 'heavy' microsomes, showing that these consist largely of particles about 15 m$\mu$ in diameter, very similar to the ribosomes obtained from animal tissues. The particles usually occur in chains or groups, and a dense body, 30–100 m$\mu$ in diameter, is frequently associated with each group. Vesicles are very rarely found in preparations of 'heavy' microsomes.

The 'light' microsomes (Plate 1, B and C) vary in appearance in different regions of the pellet. Densely packed zones (B) differ from 'heavy' microsomes in the presence of large numbers of vesicles, 50 m$\mu$ or more in diameter. More open zones (C) show very much larger vesicles, some dense bodies and few particles. The particles occur in chains, frequently attached to the dense bodies. The vesicles are 'smooth-surfaced'. In some cases there is a continuous gradation of density between smaller vesicles devoid of contents and the dense bodies, but usually the two are distinct. No systematic differences between the different root segments were found, but the vesicles of the third segment seemed usually to be larger and more numerous than those of the second. 'Light' microsomes of the first segment, obtained in very small amounts, were lost during fixation.

Hodge, Martin & Morton (1957) have also described dense bodies isolated from silver beet, but they found no association of dense bodies with ribosomes.

Fraction X was found to consist of a mixture of vesicular material, including small mitochondria-like particles and some vesicles similar to those found in 'light' microsomes. Ribonucleoprotein-like particles were very rarely found, and this

## EXPLANATION OF PLATE 1

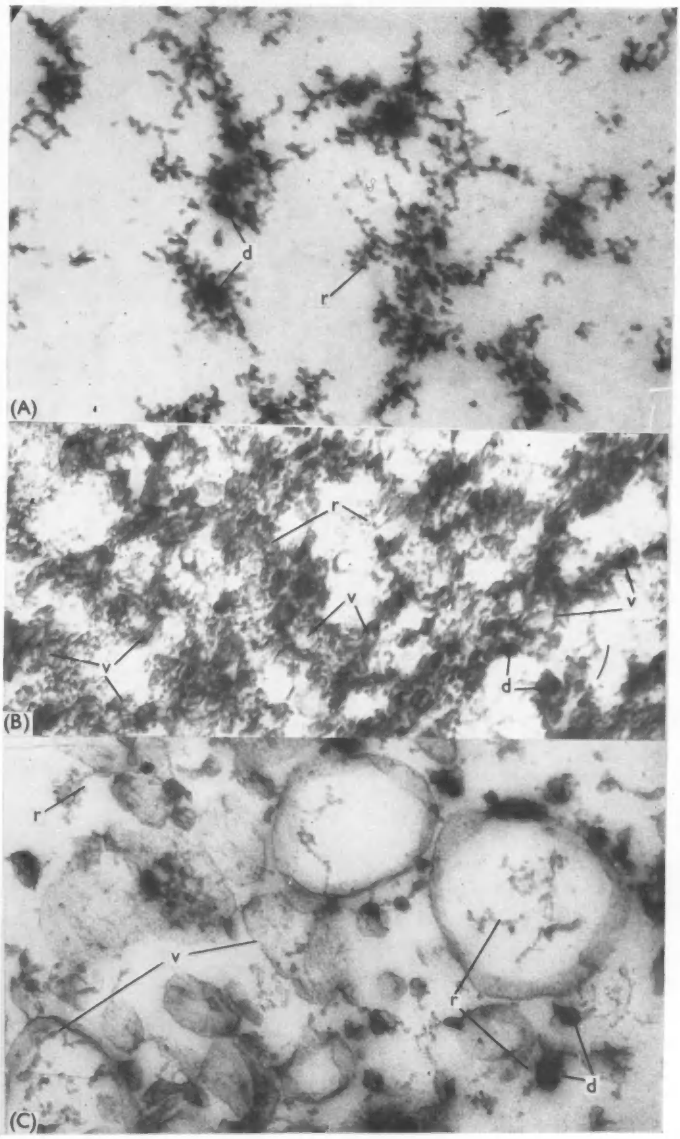
Electron micrographs of fixed and sectioned microsomal pellets ( × 50 000 approximately).

(A) 'Heavy' microsomes isolated from 6 mm. root tips, showing ribosomes (r) 15 m$\mu$ in diameter, frequently occurring in clusters in association with dense bodies (d).

(B) A densely packed region of a 'light' microsome pellet, consisting of vesicles (v) in addition to dense bodies (d) and ribosomes (r).

(C) A more open region of a 'light' microsome pellet showing larger vesicles (v) and a few ribosomes (r) and dense bodies (d).

(B) is from the second root segment and (C) from the third, but both are representative of regions in either pellet.


d
r
(A)
r
v
v
v
d
(B)
r
v
r
d
(C)

fraction is thus quite distinct from the 'heavy' microsomes. This again indicates that the centrifuging procedure was satisfactory.

## DISCUSSION

The experiments described show that a particulate and a more membranous fraction of microsomes may be separately isolated from root tips without the use of deoxycholate. Quantitative measurement of these isolated fractions shows that the proportion of membranous material increases with the age of the cell. This confirms the trend indicated by Lund, Vatter & Hanson (1958) and others, but the use of small tip segments has shown that most of the change occurs immediately before the zone of rapid cell elongation.

The separation into the 'light' and 'heavy' microsomes is sharp and reproducible, and the membranes and particles of the 'light' fraction cannot be further separated by the same techniques. It seems therefore that a true fractionation into two microsomal components has been obtained, and this is confirmed by the electron microscopy and electrophoresis. Preliminary experiments have also shown that the 'light' microsomes have a much higher reduced DPN–cytochrome *c* reductase activity than the 'heavy', in agreement with the findings of Palade & Siekevitz (1956) and others that this activity is located in the membranous fraction. The electrophoresis of the ribonuclease-treated microsomes also suggests that the ribonucleoprotein particles present in the 'light' microsomes are in some way different from those in the 'heavy'. It is possible that fraction X includes some 'light' microsomal vesicles, as indicated by the electron microscopy. The yields of fraction X were small, but if added to the yields of 'light' microsomes they would accentuate the results presented in this paper.

It is clear that the morphology and origin of these plant microsomes require further study. Electron micrographs showing the structure of the cytoplasm of young but not of mature cells have been published (e.g. Whaley *et al.* 1960). Evidence indicating which structures give rise to isolated microsomes after homogenization and centrifuging is, however, inconclusive. Nevertheless the results obtained here indicate that there is a synthesis of a membrane and particle complex at the onset of differentiation but not during the subsequent phase of cell expansion. This complex may be regarded as comparable with rough-surfaced vesicles obtained from animal tissues, although the detailed structure is different.

The plasmalemma and tonoplast do not seem to contribute to the isolated microsomes, since there is little increase in the yield when the cells expand. It is possible, however, that the smooth-surfaced vesicles obtained in the 'light' microsomes from the third root segment arise from a breakdown of the cell membranes, whereas similar vesicles from the second segment arise from the precursors of these membranes.

## SUMMARY

1. Microsomes were extracted from small serial segments of seedling pea-root tips by homogenization and centrifuging. A 'light' microme pellet consisting of a mixture of vesicles and ribosomes could be slid off the microsomal pellet, leaving a small firm pellet of 'heavy' microsomes consisting largely of ribosomes.

2. The protein and ribonucleic acid contents of the two fractions were determined and expressed as the yield per cell. The meristematic tip segment contained largely 'heavy' microsomes, and the yield of these fell with the age and maturation of the tissue. The yield of 'light' microsomes, however, increased rapidly at the earliest stages of differentiation.

3. The ribonucleic acid to protein ratio in the 'heavy' microsomes was above 1·0 and increased with the age of the tissue, whereas the ratio in the 'light' microsomes was below 0·8 and decreased with age.

4. Electrophoresis of the two fractions after disruption with ribonuclease gave more nucleic acid fragments and proteins in the 'light' microsomes than in the 'heavy'.

5. Electron micrographs of the two fractions are presented.

6. It is suggested that the synthesis of 'light' microsomes is associated with the onset of differentiation.

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# New Pigments Derived from Vitamin $B_{12}$ by a Strain of *Aerobacter aerogenes* Present in River Mud

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Micro-organisms have proved very useful in producing not only vitamin $B_{12}$, but also vitamin $B_{12}$ analogues and some of their derivatives. Thus a number of vitamin $B_{12}$ analogues containing either purines or benziminazoles have been prepared by guided biosynthesis with a vitamin-$B_{12}$-requiring mutant of *Escherichia coli* (Ford, Holdsworth & Kon, 1955; Bernhauer & Friedrich, 1954). As a result of microbial activity a number of vitamin $B_{12}$ analogues have been isolated from the gut and rumen contents, faeces of ruminants (Kon, 1955; Porter, 1957; Dion, Calkins & Pfiffner, 1952; Lewis, Tappan & Elvehjem, 1952*a*, *b*; Brown, Cain, Gant, Parker & Smith, 1955) and from sewage sludge (Friedrich & Bernhauer, 1953; Neujahr, 1956).

Phosphorylated factor B and guanosine diphosphate factor B have been isolated from cultures of *Nocardia rugosa* (Barchielli *et al.* 1960). A number of intermediates in the biosynthesis of vitamin $B_{12}$ have been isolated from *Propionibacterium shermanii* (Bernhauer, Becher, Gross & Wilharm, 1960); these include nucleotide-free carboxylic acids (amides of cobinic acid) and vitamin $B_{12}$ carboxylic acids. A new group of coenzymes, which are derivatives of vitamin $B_{12}$ or certain analogues, have been prepared from *Clostridium tetanomorphum* (Barker *et al.* 1960*a*). The vitamin $B_{12}$ coenzyme has also been prepared from *P. shermanii* (Barker *et al.* 1960*b*).

Compared with the work quoted above surprisingly little has been reported on the action of micro-organisms on vitamin $B_{12}$ and its analogues. Ford & Porter (1953) have shown that organisms present in calf faeces will induce interconversion between analogues, and micro-organisms from rotten fish were shown to degrade vitamin $B_{12}$ to unidentified products (Mori, Hashimato & Malda, 1953, 1954; Mori & Malda, 1955). In the present work the action of *Aerobacter aerogenes* on vitamin $B_{12}$ is described.

A preliminary account of some of this work has already been given (Helgeland, Jonsen & Laland, 1959).

## MATERIALS AND METHODS

*Vitamin* $B_{12}$. This was a gift from Glaxo Laboratories Ltd., Greenford, Middlesex.

[$^{58}$Co]*Vitamin* $B_{12}$. this was purchased from The Radiochemical Centre, Amersham, Bucks., and had a specific activity of 0·22 $\mu$c/$\mu$g.

*Media.* The synthetic medium contained: $NH_4Cl$, 2 g., $Na_2HPO_4,2H_2O$, 6 g.; $KH_2PO_4$, 3 g.; NaCl, 3 g.; $MgCl_2,6H_2O$, 0·04 g.; $Na_2SO_4$, 0·14 g.; water to 900 ml. The pH was adjusted to 7·2 with 10% NaOH before autoclaving. Depending on the experimental conditions, to 9 vol. of media was added either (*a*) 1 vol. of sterile water or (*b*) 1 vol. of a boiled solution containing glucose and vitamin $B_{12}$ or vitamin $B_{12}$ only.

The meat-extract medium contained: beef extract (Difco), 1·5 g.; peptone (Difco) 5 g.; $Na_2HPO_4,2H_2O$, 1 g.; water to a total volume of 500 ml. The pH of the solution was adjusted to 7·8 with 10% NaOH before autoclaving. When required, 0·1 mg. of vitamin $B_{12}$/ml. was added before autoclaving.

The agar medium contained: meat extract (Difco), 9 g.; peptone (Difco), 30 g.; $Na_2HPO_4,2H_2O$, 6·76 g.; NaCl, 9 g.; water to 1500 ml. The pH of the solution was adjusted to 8·8 with 10% NaOH and agar (37·5 g.) was added before autoclaving.

*Subculturing of micro-organism obtained from river mud.* A portion (0·1 ml.) of a suspension in 0·9% NaCl solution of mud obtained from a stream in the suburbs of Oslo was used to inoculate 3 ml. of the synthetic medium containing 0·34% (w/v) of glucose and 0·3 mg. of vitamin $B_{12}$.

Incubation was carried out in a test tube (1 cm. × 10 cm.) for 5 days at 37°. The micro-organisms were subcultured in a medium and in a manner identical with that described above.

*Growth of other bacteria in the presence of vitamin $B_{12}$.* The medium used consisted of: $FeSO_4,7H_2O$, 0·75 mg.; $CuSO_4,5H_2O$, 7·5 mg.; $ZnSO_4,7H_2O$, 7·5 mg.; $MnSO_4,4H_2O$, 75 mg.; $CaCl_2,2H_2O$, 37·5 mg.; $MgSO_4$, 0·3 g.; $KH_2PO_4$, 0·75 g.; $(NH_4)_2SO_4$, 3 g.; yeast extract (Difco), 3 g.; glucose, 0·75 g.; water to 1·5 l. Test tubes (1 cm. × 10 cm.) containing 3 ml. of medium were inoculated with one of the following bacteria, which were freshly isolated laboratory strains: *Bacillus subtilis*, *B. cereus*, *Streptococcus faecalis*, *Sarcina lutea* or *Staphylococcus aureus* H. The tubes were incubated for 12 days at 37°.

*Bacterial growth.* This was measured in a Beckman spectrophotometer at 650 mμ.

*Extraction of incubation mixtures.* Unchanged vitamin $B_{12}$ and pigments derived from the vitamin were extracted from the medium after removal of the cells by centrifuging, by brief shaking with 0·5 g. of phenol (Laland & Klem, 1936). The phenol layer, which contained all the colour, was removed after centrifuging the mixture and washed twice with 3 vol. of water to remove salts (a procedure which did not remove any colour). To the phenol layer was then added 6 vol. of ether and 1 vol. of water. After mixing and separation of the two phases by centrifuging, the coloured aqueous layer was washed twice with 2 vol. of ether and freeze-dried, and the solids were dissolved in an appropriate amount of water for further purification.

*Paper chromatography.* Descending chromatography on Whatman no. 1 paper at 25° was used. The solvent systems were butan-2-ol saturated with water–acetic acid–KCN (9·9:0·1, v/v, with 10 mg. of KCN/100 ml.) and butan-2-ol saturated with water–$NH_3$ solution (sp.gr. 0·88)–KCN (9·9:0·1, v/v, with 10 mg. of KCN/100 ml.) (Kon, 1955).

*Paper electrophoresis.* The apparatus described by Durrum (1951) was used with a voltage gradient of 10–12 v/cm., usually applied for 5–7 hr. on Whatman no. 1 or no. 3 MM paper. The following buffers were used: (1) N-acetic acid containing 0·01% of KCN, pH about 2; (2) 0·05 M-phosphate (a mixture of $KH_2PO_4$ and $K_2HPO_4$) containing 0·01% of KCN, pH 6·5; (3) 0·1 M-KCN, pH about 10.

*Absorption spectra.* These were determined in water and in 0·1 M-KCN in a Beckman spectrophotometer with photomultiplier.

*Measurement of radioactivity.* The activity of radioactive paper strips was measured by cutting out 1 cm. × 2 cm. pieces and counting these on aluminium disks in a thin end-window Geiger–Müller counter.

Radioactive solutions were plated and counted on aluminium disks at infinite thinness with a Tracerlab flow counter. Counting was continued until the count was 3000.

## RESULTS

### *Microbiological preparation of pigments derived from vitamin $B_{12}$: their electrophoretic and chromatographic properties*

Five tubes as shown in Table 1 were inoculated with a suspension in 0·9% NaCl solution of mud obtained from a stream. Phenol-extractable material from the incubation mixtures was examined by paper electrophoresis in acetic acid and by paper chromatography in butan-2-ol saturated with water–acetic acid–KCN. On electrophoresis, the extracts from tubes no. 1 and 4 gave four coloured zones, which moved towards the cathode. These were aquocobalamin, a brownish-yellow pigment (pigment I), a yellow pigment (pigment II) and unchanged vitamin $B_{12}$. Pigments I and II had mobilities 0·78 and 0·33 respectively relative to that of aquocobalamin. Paper chromatography of the same extracts gave aquocobalamin, $R_F$ 0·09, unchanged vitamin $B_{12}$, $R_F$ 0·25, a brownish-yellow pigment (pigment I), $R_F$ 0·35, and a yellow pigment (pigment II), $R_F$ 0·39.

The pigments I and II were not formed when vitamin $B_{12}$ was omitted (Table 1).

Pigments I and II isolated by direct paper chromatography of the extracts corresponded, when run electrophoretically, to the similarly coloured spots obtained by direct electrophoresis of the extracts.

The presence of aquocobalamin on the electrophoretic and chromatographic strips was shown by its spectrum in water (Veer, Edelhausen, Wijmenga & Lens, 1950). Further, 0·1 M-KCN converted it into vitamin $B_{12}$, as indicated by spectral and chromatographic properties.

When extract from tube no. 1 (Table 1) was run electrophoretically in phosphate buffer at pH 6·5, a negatively charged brownish-yellow zone (mobility

Table 1. *Conditions for formation of pigments I and II*

Tubes (1 cm. × 10 cm.) containing 3 ml. of medium were inoculated with 0·05 ml. of a suspension in 0·9% NaCl solution of mud from a stream and incubated for 5 days at 37°. The presence of pigments I and II in the material extractable with phenol from cell-free incubation mixture were detected after paper electrophoresis in N-acetic acid.

| Tube no. | Medium | Glucose (%, w/v) | Vitamin $B_{12}$ (mg.) | Bacterial growth | Formation of pigments I and II |
|---|---|---|---|---|---|
| 1 | Synthetic | 0·34 | 0·3 | + | + |
| 2 | Synthetic | 0·34 | 0 | + | − |
| 3 | Synthetic | 0 | 0·3 | − | − |
| 4 | Beef extract–peptone | 0 | 0·3 | + | + |
| 5 | Beef extract–peptone | 0 | 0 | + | − |

0·85 relative to aquocobalamin), which separated into pigments I and II when run in butan-2-ol saturated with water–acetic acid–KCN, was obtained. In addition aquocobalamin with one positive charge, unchanged vitamin $B_{12}$ with no charge, and a pink negatively charged zone were detected. This latter unknown zone was not further investigated.

In 0·1 M-KCN pigments I and II had the same mobility as vitamin $B_{12}$.

*Absorption spectra*

Pigments I and II were purified by repeated (twice) paper chromatography in butan-2-ol saturated with water–acetic acid–KCN followed by paper electrophoresis in acetic acid. The two substances purified in this manner were stored in the freeze-dried state.

In recording the spectra (Fig. 1) for pigment I, molecular extinction coefficients are used. These could, as shown later, be calculated by using [$^{58}$Co]vitamin $B_{12}$ for the preparation of pigment I. For pigment II, extinction values only are recorded (Fig. 2).

In Fig. 1 a comparison is made between the spectra of pigment I and vitamin $B_{12}$ in water. Pigment I exhibits a distinct peak in the ultraviolet region at 262 m$\mu$, somewhat displaced towards lower wavelength as compared with the vitamin (278 m$\mu$). The most conspicuous feature is, however, the small peak at 342 m$\mu$ as compared with the large peak in the 300–400 m$\mu$ region for vitamin $B_{12}$. For pigment I the peak in the visible region occurs at 474 m$\mu$.

When pigment I (brownish-yellow) was dissolved in 0·1 M-KCN, the solution turned pink with a pronounced bathochromic shift of the maximum from 474 to 500 m$\mu$ (Fig. 1). This property is similar to that of vitamin $B_{12}$, which also exhibits a bathochromic shift in KCN with a concomitant change in colour. On acidifying with acetic acid the colour changed back to brownish-yellow and pigment I was reisolated by paper chromatography and paper electrophoresis.

The noticeable feature in the spectrum of pigment II (Fig. 2) in water is the absence of a large peak in the 300–400 m$\mu$ region. When dissolved in 0·1 M-KCN there was a bathochromic shift in the maximum from 448 to 482 m$\mu$.

The bathochromic shift in the spectra of pigments I and II in KCN was probably due to complex-formation (as with vitamin $B_{12}$) with cyanide and not merely to a change in pH, since no such shift was experienced when to aqueous solutions of pigments I and II was added $NH_3$ to the same pH (10).

*Presence of cobalt in pigment I*

Synthetic medium (3 ml.) containing 0·34 % of glucose, 1·2 $\mu$g. of [$^{58}$Co]vitamin $B_{12}$ (6920 counts/min.) and 0·3 mg. of inactive vitamin $B_{12}$ was inoculated with organisms subcultured five times in the synthetic medium containing glucose and vitamin $B_{12}$. The radioactive culture was incubated for 5 days at 37°, and pigment I isolated from the phenol-extractable material by paper electrophoresis in acetic acid and further purified by

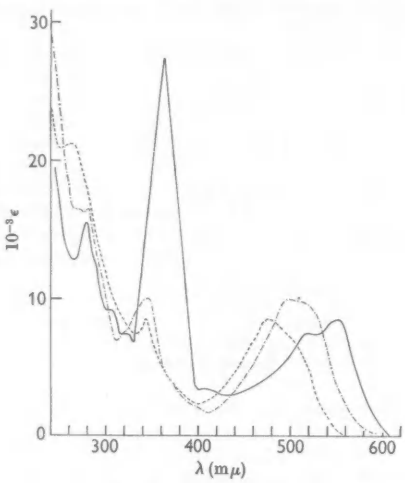


Fig. 1. Absorption spectra of vitamin $B_{12}$ in water (——), and pigment I in water (- - - -) and in 0·1 M-KCN( — · —).

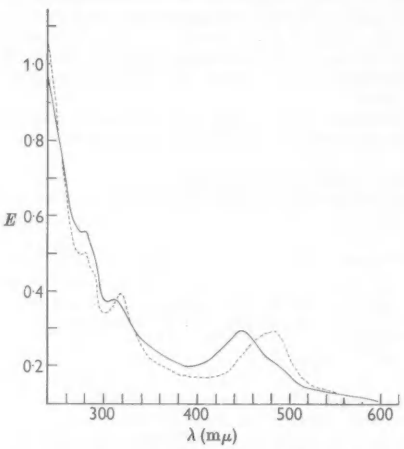


Fig. 2. Absorption spectra of pigment II in water (——), and in 0·1 M-KCN (- - - - -).

paper chromatography in butan-2-ol saturated with water–acetic acid–KCN. The radioactivity along the strip was counted. At this stage of purification some vitamin $B_{12}$ was present and probably originated from contaminating aquocobalamin, which runs close to pigment I in the initial electrophoretic separation. The radioactive area containing pigment I was purified further by paper chromatography in butan-2-ol saturated with water–$NH_3$ solution–KCN. To exclude the possibility that some radioactive vitamin $B_{12}$ was still only loosely adsorbed to pigment I, 0·05 mg. of inactive vitamin $B_{12}$ was mixed with the eluted radioactive pigment I and the mixture submitted to electrophoresis in acetic acid. Examination of the electrophoretic strip revealed that pigment I retained its radioactivity and therefore contains cobalt.

*Molecular extinction coefficient of pigment I*

An incubation mixture similar to that described above was prepared in duplicate. By using the light absorption at 548 m$\mu$ to calculate the amount of vitamin $B_{12}$ present, the average specific activity of vitamin $B_{12}$ in the two solutions was determined before incubation (65·5 counts/min./$\mu$g.). The phenol-extractable material obtained after incubation from the two incubation mixtures was purified by electrophoresis in N-acetic acid. Pigment I was subsequently purified by paper chromatography in butan-2-ol saturated with water–acetic acid–KCN, eluted and freeze-dried. The material was dissolved in 1 ml. and the absorption at 474 m$\mu$ determined; 0·1 ml. was used for radioactivity measurements. Assuming one atom of cobalt per molecule the molecular extinction coefficient was calculated for the two duplicates ($7{\cdot}9 \times 10^3$ and $8{\cdot}8 \times 10^3$ respectively). The remaining material from the duplicates was combined and run in butan-2-ol saturated with water–$NH_3$ solution–KCN. Pigment I was eluted and the molecular extinction coefficient again determined ($8{\cdot}5 \times 10^3$). The mean molecular extinction coefficient at 474 m$\mu$ is therefore $8{\cdot}4 \times 10^3$ and was used for the calculations to be described.

*Formation of pigment I under different conditions*

An extract containing pigment I was obtained from the incubation mixtures by the phenol method. The pigment was separated from other material by electrophoresis in N-acetic acid. The zone of pigment I was eluted from the paper with 3 ml. of water and the absorption at 474 m$\mu$ used to calculate the amount of pigment present.

*Varying amounts of vitamin $B_{12}$.* Each tube contained 3 ml. of synthetic medium with 0·34 % (w/v) of glucose and vitamin $B_{12}$ and was incubated for 5 days at 37° after inoculation with micro-organisms subcultured seven times. There was a linear relationship between the amount of vitamin $B_{12}$ per tube and pigment I produced. The maximum amount of vitamin $B_{12}$ per tube tested was 660 $\mu$m-moles, which gave 20 $\mu$m-moles of pigment I.

*Incubation time.* The relationship between the formation of pigment I and the incubation period is shown in Fig. 3. The production of pigment I took place at a much slower rate than the bacterial growth. To obtain a maximum yield of pigment I, an incubation period of at least 12 days was required.

*Effect of glucose concentration.* The yield of pigment I was very dependent on the glucose concentration (Fig. 4). The highest yield was obtained

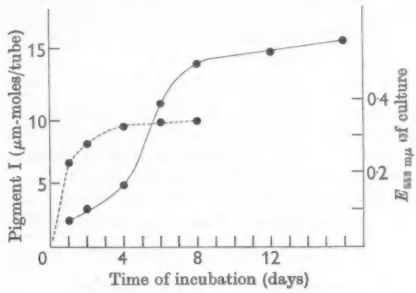


Fig. 3. Time curves for the formation of pigment I (——) and the bacterial growth (- - - - -). Each tube contained 3 ml. of synthetic medium with 0·34 % (w/v) of glucose and 200 $\mu$m-moles of vitamin $B_{12}$. The tubes were incubated at 37° after inoculation with micro-organisms subcultured seven times. The amount of pigment I formed was determined as described in the text.

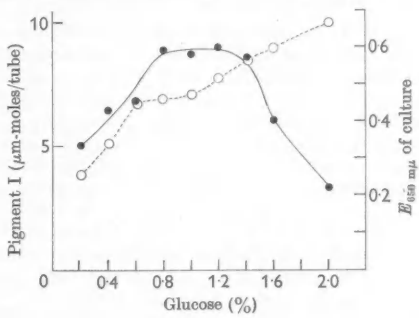


Fig. 4. Formation of pigment I (——) at different concentrations of glucose. The bacterial growth curve (- - - - -) is also recorded. Each tube contained 3 ml. of synthetic medium with 0·3 mg. of vitamin $B_{12}$ and glucose and were incubated for 5 days at 37° after inoculation with micro-organisms subcultured seven times. The amount of pigment I formed was determined as described in the text.

Table 2. *Effect of oxygen on the yield of pigment I*

Each incubation mixture consisted of 3 ml. of synthetic medium containing 0·8% (w/v) of glucose and 780 $\mu$m-moles of vitamin $B_{12}$. After inoculation with micro-organisms subcultured seven times, the mixtures were incubated at 37° for 12 days. Aerobic incubation was carried out with occasional shaking in a 10 ml. conical flask (surface area of incubation mixture 7 cm.$^2$). Anaerobic incubation was in a test tube (1 cm. × 10 cm.) in an atmosphere of hydrogen, and the partly anaerobic incubation in the same kind of test tube in the presence of oxygen (without shaking). The amount of pigment I formed was determined as described in the text.

| Conditions | Amount of pigment I formed ($\mu$m-moles) | $E_{650\ m\mu}$ of culture |
|---|---|---|
| Aerobic | 4·6 | 0·52 |
| Anaerobic | 20·6 | 0·18 |
| Partly anaerobic | 19·2 | 0·42 |

with a glucose concentration in the range 0·8–1·2 %. It is also seen that a large bacterial growth does not necessarily produce a better yield of pigment I.

*Aerobic and anaerobic formation of pigment I.* Pigment I was produced both under aerobic and anaerobic conditions, but anaerobic conditions greatly favour its formation (Table 2).

### *Identification of micro-organism*

By subcultivation of the incubation mixtures on agar plates, rough and smooth colonies of *A. aerogenes* were obtained. Transformation of vitamin $B_{12}$ was subsequently achieved by subcultures from single colonies of both the rough- and the smooth-colony type.

### *Formation of pigment I by other bacteria*

None of the following bacteria, *B. subtilis*, *B. cereus*, *Streptococcus faecalis*, *Sarcina lutea* and *Staphylococcus aureus* (Strain H), when grown on a glucose plus salts medium containing vitamin $B_{12}$ produced detectable quantities of pigment I as judged by paper electrophoresis in acetic acid of phenol-extractable material of the cell-free incubation mixture.

## DISCUSSION

The absorption spectrum of the brownish-yellow pigment I derived from vitamin $B_{12}$ exhibits a distinct peak in the ultraviolet region (Fig. 1), suggesting the presence of the nucleotide in the molecule. The absence of a large peak in the 300–400 m$\mu$ region in pigment I as compared with vitamin $B_{12}$ is also found in other vitamin $B_{12}$ derivatives such as Beaven & Johnson's (1955) brownish reduction product, breakdown products of vitamin $B_{12}$ produced as a result of $\gamma$-irradiation (Sjöstedt & Ericson, 1959), the cobamide coenzymes (Barker *et al.* 1960*a*, *b*), the product(s) obtained when reducing aquocobalamin catalytically in the presence of adenine (Bernhauer, Gaiser, Müller & Wagner, 1960), the solution of cobinamide reduced with sodium dithionite in the presence of adenine (Pawelkiewicz, Bartosinski & Walerych, 1960) and the solution of factor B treated with the same reducing agent (George, Irvine & Glauser, 1960). Pigment I, however, is stable towards air and daylight and is therefore different from these products.

The yield of pigment I is considerably higher under anaerobic conditions and it is therefore possible that some reduction of vitamin $B_{12}$ is involved in its formation. Several reduction products of vitamin $B_{12}$ involving the central cobalt atom are described in the literature. On catalytic hydrogenation a brownish-coloured reduction product of vitamin $B_{12}$ (Kaczka, Wolf & Folkers, 1949; Diehl & Murie, 1952; Boos, Carr & Conn, 1953; Beaven & Johnson, 1955) containing bivalent cobalt, as shown by polarographic and titrimetric evidence (Jasalkis & Diehl, 1954), is obtained. This substance is readily reoxidized to hydroxocobalamin by exposure to air. A second, more highly reduced, grey–green substance is also reoxidizable to hydroxocobalamin (Diehl & Murie, 1952; Boos *et al.* 1953; Beaven & Johnson, 1955).

A brownish-green colour is produced when vitamin $B_{12}$ is heated with alkali in the absence of air. It has been shown that a reduction in the valency of the cobalt atom accompanied by oxidation in the B-ring and its propionamide side chain has occurred (Smith, 1955; Bonnett *et al.* 1957). In this case also the cobalt atom is reoxidized upon exposure to air as shown by the return of the red colour.

The cobamide coenzymes have recently been shown to contain bivalent cobalt (Bernhauer, Gaiser, Müller, Müller & Günter, 1960; Johnson & Shaw, 1960) and are, in contrast with the reduction products described above, stable to oxygen provided no exposure to light occurs. The presence in the coenzymes of the adenine nucleoside somehow stabilizes the bivalent cobalt co-ordination complex.

Pigment I might have a structure analogous to the coenzymes on the basis of its absorption spectrum and its favoured formation under anaerobic conditions. The increased stability of pigment I in daylight under oxygen and towards potassium cyanide as compared with the coenzymes could be due to the nature of the substance which is co-ordinating with the bivalent cobalt in pigment I.

Another possible feature of the structure for pigment I, which could account for its spectrum, would be a molecule in which some interruption in the six conjugated double bonds present in vitamin $B_{12}$ had occurred.

To explain the formation of pigment I one also must take into account its positive charge in N-acetic acid–potassium cyanide (pH 2·5), and its negative charge in phosphate buffer (pH 6·5) and potassium cyanide (pH 10).

It is tentatively suggested that the formation of pigment I by *A. aerogenes* involves the reduction of the cobalt atom in vitamin $B_{12}$ followed by co-ordination of the bivalent cobalt to some ionizable substance provided by the micro-organism. The reaction could be enzymic or non-enzymic.

## SUMMARY

1. Crude cultures from river mud containing predominantly *Aerobacter aerogenes* produces a brownish-yellow pigment (pigment I) and a yellow pigment (pigment II) when grown in the presence of vitamin $B_{12}$ in a synthetic salt–glucose medium or a meat-extract medium. Pure cultures of both the rough and the smooth type of *Aerobacter aerogenes* were also shown to possess this property.

2. Both pigments are derived from vitamin $B_{12}$. Pigment I has been shown to contain cobalt. It is positively charged at pH 2·5 and negatively charged at 6·5. It has a distinct peak in the ultraviolet region at 262 m$\mu$ and lacks the large peak in the 300–400 m$\mu$ region characteristic of vitamin $B_{12}$. In potassium cyanide the pigment turns pink and there is a bathochromic shift in the spectrum. This reaction is reversible. Pigment II is also positively charged at pH 2·5 and negatively charged at pH 6·5 and lacks the large peak in the 300–400 m$\mu$ region.

3. Within certain limits the formation of pigment I increases linearly with the concentration of vitamin $B_{12}$. The formation of this substance is very slow and requires 12 days to obtain maximum yield. Anaerobic conditions greatly favour its formation, and the yield is also very dependent on the glucose concentration, reaching a maximum at a glucose concentration in the region 0·8–1·2 %.

4. No detectable formation of pigment I and II was achieved with the following bacteria: *Bacillus subtilis*, *B. cereus*, *Streptococcus faecalis*, *Sarcina lutea* and *Staphylococcus aureus* H.

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# The Synthesis of Sucrose by Extracts of the Root of the Sugar Beet

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There is no doubt that synthesis of sucrose does occur in the leaf of the sugar beet but the root has not previously been shown to possess a sucrose-synthesizing system.

Buchanan *et al.* (1952) and Buchanan (1953), in photosynthetic experiments with $^{14}CO_2$, demonstrated the formation of sucrose phosphate in the leaves of the sugar beet as well as of other plants. They postulated that the sucrose phosphate was formed according to (1):

$$\text{UDPG} + \text{fructose 1-phosphate} \rightleftharpoons \text{sucrose phosphate} + \text{UDP} \quad (1)$$

Cardini, Leloir & Chiriboga (1955) reported that extracts of wheat germ and other plant materials would bring about the synthesis of sucrose according to (2):

$$\text{UDPG} + \text{fructose} \rightleftharpoons \text{sucrose} + \text{UDP} \quad (2)$$

Negative or non-reproducible results were obtained, however, with extracts of sugar-beet leaves or roots.

Burma & Mortimer (1956), using [$^{14}$C]glucose 1-phosphate, showed that sugar-beet-leaf homogenates could synthesize uridine diphosphate glucose (UDPG) from uridine triphosphate and glucose 1-phosphate. The same homogenates yielded sucrose and traces of sucrose phosphate from uridine diphosphate glucose and fructose 6-phosphate and the authors considered that the sucrose phosphate was the initial product:

$$\text{UDPG} + \text{fructose 6-phosphate} \rightleftharpoons \text{sucrose phosphate} + \text{UDP} \quad (3)$$

and that the sucrose was formed subsequently by dephosphorylation. Sucrose was not produced if fructose 6-phosphate was replaced by fructose or by fructose 1:6-diphosphate.

More recently, Rorem, Walker & McCready (1960) have demonstrated the presence of enzymes capable of catalysing both reactions (2) and (3) in sugar-beet-leaf extracts from which invertase had been partially eliminated.

Kursanov & Pavlinova (1952) and Kursanov (1954) found that sucrose was produced when glucose and fructose were introduced into leaf disks by vacuum infiltration, but they were unable to detect any sucrose synthesis in similar infiltration experiments with root samples, except possibly to a slight extent with young roots.

It is now demonstrated that the necessary precursors and an enzyme system for the synthesis of sucrose are present in the sugar-beet root.

## EXPERIMENTAL

*Materials.* Adenosine monophosphate (AMP), adenosine diphosphate (ADP) and fructose 6-phosphate (F6-P) were obtained from L. Light and Co. Ltd. Adenosine triphosphate (ATP), glucose 1-phosphate (G1-P) and glucose 6-phosphate (G6-P) were obtained from British Drug Houses Ltd. Uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP) and uridine diphosphate glucose (UDPG) were obtained from Sigma Chemical Company, St Louis, Mo., U.S.A.

*Preparation of extracts.* Healthy beet plants, which had been sown outdoors in April, were harvested at intervals from June to November. They were thoroughly washed with cold water and extracted, usually within 1 hr. of harvesting.

The sample of plant material was chopped and 200 g. was macerated in 250 ml. of ice-cold 0·05 M-phosphate ($Na_2HPO_4$–$KH_2PO_4$) buffer, pH 7·0. The macerate was filtered rapidly, first on stainless-steel gauze and then through glass wool, to yield 150 ml. of crude extract, which was immediately centrifuged at 34 000*g* for 5 min. The precipitate was discarded and the supernatant was saturated with $(NH_4)_2SO_4$. The solution was allowed to stand in a refrigerator for 1 hr. and then centrifuged again at 34 000*g* for 5 min. The supernatant was discarded and the precipitate was taken up in the minimum of water and dialysed against 0·05 M-phosphate buffer, pH 7·0, at 5° for 24 hr. Approx. 10–15 ml. of concentrate, free from sucrose and nucleotides, was obtained. The extract was used within 48 hr. since the activity diminished on storage at 5°. Freezing caused coagulation and complete loss of sucrose-synthesizing activity.

Throughout the extraction, temperatures above 10° were normally avoided except during centrifuging, but temperature control was not critical and active extracts could be prepared without refrigeration.

*Detection of sucrose-forming activity.* The digests contained: 0·02 ml. of 2·0 M-tris–HCl buffer, pH 7·2; 1·5 $\mu$moles of fructose; 0·25 $\mu$mole of UDPG and 0·25 ml. of extract; total volume 0·3 ml. Two blank digests, one in which fructose was omitted, the other in which UDPG was omitted, were also prepared.

After 30 min. at 37° the reaction was terminated and residual fructose was destroyed, as described by Cardini *et al.* (1955); sucrose was estimated by a modification of the resorcinol method of Roe (1934), as described by Bacon & Bell (1948). After incubation, 0·20 ml. of 0·5 N-NaOH solution was added to each digest and, after careful mixing, the tubes were heated for 10 min. in a boiling-water bath. The tubes were cooled, 0·5 ml. of water and 1·5 ml. of

resorcinol solution (0·15%, w/v, in absolute ethanol) were added, followed 1 min. later by 1·5 ml. of conc. HCl (containing 7·5 mg. of $FeCl_3$/l.). The tubes were heated for 30 min. in a thermostatically controlled water bath at 75°. After cooling, the colours were measured at 490 mμ and the sucrose content was calculated from the extinction of concurrent sucrose standards.

Higher concentrations of UDPG and fructose were employed in preparative experiments and also in experiments designed to estimate both sucrose synthesis and changes in nucleotide composition. In these experiments the reaction was terminated by the addition of 1 vol. of ethanol. The clear supernatant obtained after centrifuging was used for determining nucleotides and sucrose. In these extracts, sucrose was separated by paper chromatography, detected with the α-naphthol reagent, and occasionally estimated by visual comparison with the colour developed by known amounts of sucrose on the same chromatogram. Results obtained by this method were consistent with those obtained by the resorcinol method.

*Estimation of nucleotides.* Nucleotides were estimated spectrophotometrically after separation by paper chromatography and detection by contact photography by using a Hanovia 253·7 mμ ultraviolet source (Hanovia Ltd., Slough, Bucks.). The areas containing ultraviolet-absorbing material were cut from the chromatograms with appropriate blanks and were soaked in portions (5 ml.) of 0·1 N-HCl. Nucleotide concentration was determined from the extinction. A molar extinction coefficient of $15 \times 10^3$ at 257 mμ was used for adenosine compounds and $10 \times 10^3$ at 262 mμ for uridine compounds.

*Paper chromatography and electrophoresis.* All separations were carried out on Whatman no. 3MM paper. Solvents 1–6 (proportions by vol.) were employed for the separation of sugars: (1) propan-1-ol–ethyl acetate–water (7:1:2); (2) ethyl acetate–acetic acid–water (3:1:3); (3) propan-1-ol–aq. $NH_3$ (sp.gr. 0·88)–water (80:5:15); (4) butan-1-ol–ethanol–water (10:1:2); (5) butan-1-ol–acetic acid–water (3:3:2); (6) propan-1-ol–benzyl alcohol–formic acid–water (50:72:17:23).

Nucleotides were separated by chromatography in the solvents: (7) ethanol–M-ammonium acetate soln., pH 7·5 (5:2); (8) *iso*butyric acid–aq. $NH_3$ (sp.gr. 0·88)–water (66:1:33) and by high-voltage paper electrophoresis (100 v/cm.) in citrate buffer, pH 3·3 (0·2 M-citric acid–0·2 M sodium citrate, 3:1).

*Spray reagents.* Fructose and sucrose were detected with α-naphthol–phosphoric acid (Albon & Gross, 1950) and urea–phosphoric acid (Wise, Dimler, Davis & Rist, 1955). Glucose and fructose were detected with $AgNO_3$–NaOH (Trevelyan, Procter & Harrison, 1950). Glucose and G 6-P were detected as brown spots and G 1-P as a yellow spot with *p*-anisidine–phosphoric acid (Mukherjee & Srivastava, 1952); UDPG also reacted weakly to give a yellow spot. After electrophoresis in 0·1 M-borate buffer, glucose phosphates were detected by spraying with $FeCl_3$–salicylsulphonic acid (Wade & Morgan, 1953) modified by addition of 1·75 ml. of conc. HCl/100 ml. of $FeCl_3$ soln. to counteract the effect of residual buffer.

## RESULTS

*Distribution of acid-soluble nucleotides.* An initial examination was undertaken principally to determine whether UDPG was present in sugar beet and whether there was any differential concentration between particular sections. The plants were divided into blade, petiole, crown portion of the root and residual main root, and the four sections were extracted by the technique described by Bergkvist (1956) for the isolation of the acid-soluble nucleotides of wheat plants.

UDPG was identified in all the extracts and the concentration, which was similar in all four sections, was of the order of 1 μmole/100 g. of fresh plant material. UDP, ADP and ATP were also present in all extracts, but no UTP was detected.

*Synthesis of sucrose.* When enzyme extracts from the above-mentioned plant sections were incubated with UDPG and fructose, no resorcinol reaction for sucrose was obtained with leaf or stem extracts but crown and root extracts gave positive results. When fructose was replaced by fructose 6-phosphate negative results were obtained with all enzyme extracts. The activity of root and crown extracts when incubated with UDPG and fructose appeared to be similar, so that all subsequent experiments were carried out with extracts prepared from the whole root including the crown.

The resorcinol-positive compound was identified as sucrose by paper chromatography and by hydrolysis to glucose and fructose. Digests, after incubation at 37°, were treated with an equal volume of ethanol and centrifuged. The supernatant solutions were applied to paper chromatograms, which were developed in solvents 1–6. Chromatograms were sprayed with the α-naphthol or the urea reagent. Blank digests containing UDPG plus root extract gave no reaction; those containing fructose plus root extract gave only a fructose spot whereas digests containing UDPG, fructose and root extract gave, together with the fructose spot, a slower-moving spot. This product when mixed with sucrose gave a single spot on chromatograms in all six solvents and, like sucrose, gave a violet reaction with α-naphthol, blue with urea and a very weak reaction with the silver nitrate-spray test.

Digests containing approx. 0·3 μmole of the product were applied as bands to paper chromatograms, which were then irrigated in solvent 1 for 40 hr. The positions of the fructose band and the slower-moving band were established by spraying guide strips with the α-naphthol reagent. The slower-moving band was cut from the unsprayed section of the chromatogram and eluted with water to give approx. 0·5 ml. of eluate. One-half of this eluate was acidified with 0·05 ml. of 0·1 N-hydrochloric acid and placed, together with the remaining untreated eluate, in a boiling-water bath for 15 min. The acid-treated eluate was neutralized with 0·1 N-sodium hydroxide soln. and both eluates were applied to paper chromatograms,

which were developed in solvents 2, 4 and 6. The sugars were detected by spraying the chromatograms with silver nitrate–sodium hydroxide soln. The untreated eluate was found to contain only sucrose, whereas in the acid-treated eluate sucrose had disappeared and two products had been formed which, when mixed with glucose and fructose respectively, gave single spots.

In a similar experiment one-half of the eluate was treated with 0·01 ml. of invertase concentrate (Sumasuco, The Sugar Manufacturers' Supply Co. Ltd.) and together with the untreated portion of eluate was incubated at 45° for 6 hr. Again paper chromatography showed sucrose in the untreated eluate and glucose and fructose together with a trace of residual sucrose in the invertase-treated eluate.

Although no sucrose was detected if blade or petiole extracts were incubated under the standard conditions, sucrose was detected chromatographically with the petiole extract when the quantity of UDPG in the digest was increased 20-fold. Even with the increased UDPG concentration, negative results were still obtained with blade extracts.

If the sucrose is formed according to the reaction

$$\text{UDPG} + \text{fructose} \rightleftharpoons \text{sucrose} + \text{UDP}, \qquad (2)$$

the nucleotide product, UDP, should also be detectable.

Digests in which sucrose had been synthesized were found to contain an ultraviolet-absorbing compound which was identical with authentic UDP on paper chromatograms and electrophoretograms.

The yield of UDP was always less than the yield of sucrose but UMP and uridine were also formed. Traces of UMP were present as impurity in the UDPG before incubation. It therefore appeared that UDPG and UDP were being degraded, probably by nucleotide pyrophosphatase and nucleotidase. Degradation was found to be particularly rapid with root extracts that had been dialysed against water instead of the recommended phosphate buffer. Although such extracts synthesized sucrose from UDPG and fructose, the yield of sucrose was occasionally so low that it could not be detected by paper chromatography but only by the resorcinol method. In these extracts UDPG was found to be completely degraded, sometimes after incubation for only 30 min. at 37°.

On incubation of the digests without fructose, UDPG was degraded to UMP and not UDP, suggesting the simultaneous production of a glucose phosphate. These digests were applied to paper chromatograms and the products were separated by development in solvent 7. The nucleotides were characterized by ultraviolet photography and the glucose derivatives were detected with the *p*-anisidine reagent. The glucose phosphate produced initially gave a single spot on chromatograms with, and the same yellow reaction as, glucose 1-phosphate. With increasing time of incubation a second product, running just behind glucose 1-phosphate and giving a red–brown reaction, was observed. This was found to give a single spot on chromatograms with glucose 6-phosphate. The identification of glucose 1-phosphate and glucose 6-phosphate was confirmed by electrophoresis (100 v/cm.) in 0·1 M-borate, pH 9·5. No evidence for the cyclic glucose phosphate, reported by Paladini & Leloir (1952) to arise by alkaline degradation of UDPG, was obtained. The presence of phosphoglucomutase in the extract was confirmed by incubating with either glucose 1-phosphate or glucose 6-phosphate, when their interconversion was detected by paper chromatography and electrophoresis.

Sucrose synthesis ceased with the complete degradation of UDPG in the digests and attempts to synthesize sucrose by incubating root extract and glucose 1-phosphate with fructose or fructose 6-phosphate failed. It was therefore concluded that UDPG was essential for the synthesis and that this occurred according to reaction (2).

A quantitative presentation of the principal reactions described above is recorded in Fig. 1, the formation of sucrose and UDP from UDPG and fructose, together with the degradation of UDPG, UDP and UMP, being shown.

*Phosphatase action.* The comparative rates of enzymic degradation of various nucleotides were determined.

The results (Fig. 2) demonstrate that the rates of degradation decrease in the order ATP, ADP, UDP, UTP, UDPG, AMP, UMP.

In contrast, chromatography of similar digests containing 10 $\mu$moles of glucose 1-phosphate or 6 $\mu$moles of fructose 6-phosphate showed that no free glucose or free fructose was present after incubation for 4 hr.

Whereas the degradation of UTP, UDP, UMP and AMP occurs by the loss of one phosphate group and in UDPG by the loss of glucose 1-phosphate, the degradation of ADP and ATP was found to be more complex. Dilute digests were prepared, containing only 0·01 ml. of enzyme extract in 0·5 ml., and, on incubation with ADP, approximately equivalent amounts of ATP and AMP were produced, probably owing to the presence of myokinase:

$$2\,\text{ADP} \rightleftharpoons \text{ATP} + \text{AMP} \qquad (4)$$

Furthermore, the rate of production of AMP from ATP in these dilute digests was much greater than the calculated rate of production if the source

of the AMP was the ADP yielded by dephosphorylation of the ATP. It was concluded that some of the AMP was produced by apyrase activity:

$$ATP \rightleftharpoons AMP + P\text{-}P \qquad (5)$$

*Synthesis of uridine diphosphate glucose from uridine triphosphate and glucose 1-phosphate.* The examination of the acid-soluble nucleotides showed that though UDPG was present in the beet, its concentration was low. It was therefore clear that if this nucleotide were to play an important role in the synthesis of sucrose in the sugar beet, there must be enzymic pathways for its regeneration. The most likely pathway was by the reaction

$$UTP + G1\text{-}P \rightleftharpoons UDPG + P\text{-}P \qquad (6)$$

catalysed by UDPG pyrophosphorylase.

When root extracts were incubated with glucose 1-phosphate and UTP, a nucleotide product giving a single spot on chromatograms with authentic UDPG was obtained. This product was not present in blank digests in which either UTP or glucose 1-phosphate was omitted. The degradation products, UDP, UMP and uridine, were again formed in both active and blank digests containing UTP.

Electrophoresis in citrate buffer showed that the synthesized product had the same mobility as UDPG. Further confirmation of its identity was obtained by hydrolysis. Approximately 1 $\mu$mole of the product was separated from the other nucleotides by developing a band paper chromatogram in solvent 7. This effected only a partial separation between the product and glucose 1-phosphate. The strip containing the synthesized material plus contaminating glucose 1-phosphate was eluted with water and re-applied, as a band, to a second chromatogram, which was developed in 70% propan-2-ol to give complete separation of the nucleotide from glucose 1-phosphate. The nucleotide band was cut from the chromatogram and eluted with water to give approx. 0·5 ml. of eluate. A portion (0·2 ml.) of eluate was acidified with 0·02 ml. of 0·1 N-hydrochloric acid, heated at 100° for 15 min. and finally cooled and neutralized with

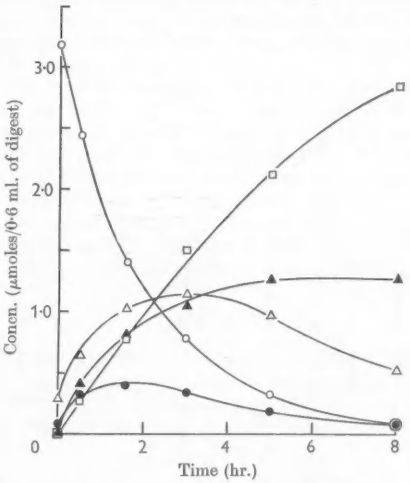


Fig. 1. Synthesis of sucrose from UDPG plus fructose by an enzyme extract of sugar-beet root. Reaction mixtures contained: 5·0 $\mu$moles of fructose, 3·18 $\mu$moles of UDPG (plus 0·29 $\mu$mole of UMP and 0·08 $\mu$mole of UDP as impurities), 0·02 ml. of 2·0 M-tris–HCl buffer, pH 7·2, 0·30 ml. of root extract; total volume 0·60 ml. Temp. 37°. Sucrose was estimated both by the resorcinol technique and by paper chromatography. UDP, UMP, UDPG and uridine were separated by paper chromatography and estimated spectrophotometrically. □, Uridine; ▲, sucrose; △, UMP; ●, UDP; ○, UDPG.

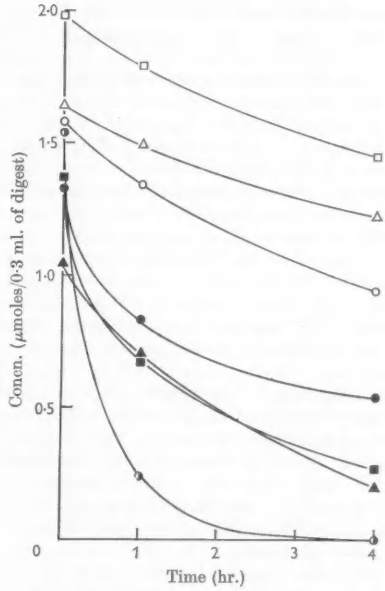


Fig. 2. Degradation of nucleotides by phosphatase in sugar-beet-root extract. Each digest contained 0·01 ml. of 2·0 M-tris–HCl buffer, pH 7·2, 1·0–2·0 $\mu$moles of nucleotide and 0·15 ml. of root extract in a total volume of 0·30 ml. After incubation at 37° the digests were inactivated by the addition of 1 vol. of ethanol. Nucleotide concentrations were determined spectrophotometrically after paper chromatography in solvents 7 and 8. □, AMP; ■, ADP; ◑, ATP; ○, UDPG; ●, UDP; △, UMP; ▲, UTP.

0·1 N-sodium hydroxide. Paper chromatography in solvents 2, 4 and 7 and electrophoresis in citrate buffer showed that the hydrolysate contained glucose, UDP and UMP, whereas only UDPG was present in the initial eluate.

The course of the synthesis of UDPG from UTP, together with the degradation of the nucleotides, is recorded in Fig. 3. Digests were inactivated by the addition of 1 vol. of ethanol. UTP, UDP, UMP, UDPG and uridine were separated by paper chromatography in solvent 7 and estimated spectrophotometrically.

*Synthesis of uridine triphosphate from adenosine triphosphate and uridine diphosphate.* Though the presence of UDPG pyrophosphorylase in the root extract could be demonstrated, this mode of regeneration of UDPG requires a source of UTP and this nucleotide was not detected in the initial examination of the beet sections. The synthesis of UTP by nucleoside-diphosphokinase activity was demonstrated by incubation of ATP and UDP with the root extract; a product identical with authentic UTP on paper chromatography or electrophoresis was rapidly obtained:

$$ATP + UDP \rightleftharpoons UTP + ADP \qquad (7)$$

The activity of this enzyme was sufficiently high to permit use of a low concentration (2 %) of root extract, whereas a higher concentration (50–67 %) was employed to demonstrate the synthesis of sucrose and UDPG. Addition of ethanol was not completely effective in stopping the reaction, which was therefore terminated by immersion of the digests in a boiling-water bath for 5 min.

UTP and ADP were formed in digests containing UDP and ATP, and no UTP was formed in blank digests from which either UDP or ATP was omitted. ADP was, however, produced together with AMP in blank digests containing ATP and root extract.

Fig. 4 illustrates the formation of UTP, together with the partial degradation of the nucleotides to the monophosphate stage. The reaction is also influenced by myokinase (4) and apyrase (5) activity, but, since the concentration of root extract was low, the phosphatase activity was minimized so that nucleosides were not produced to a detectable extent.

Nucleotides were separated for estimation in solvent 8, and whereas all other nucleotides were clearly separated the UMP spot coincided with that of ATP in this solvent. UMP was therefore estimated after separation from the other components in solvent 7 and ATP was then calculated by difference.

*Synthesis of uridine diphosphate glucose from uridine diphosphate, adenosine triphosphate and glucose 1-phosphate.* After demonstration of syn-

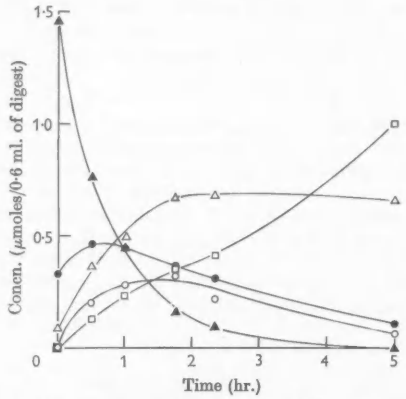


Fig. 3. Synthesis of UDPG from UTP plus glucose 1-phosphate by an enzyme extract of sugar-beet root. Reaction mixtures contained: 10·0 μmoles of glucose 1-phosphate, 1·45 μmoles of UTP (plus 0·32 μmole of UDP and 0·08 μmole of UMP as impurities), 0·02 ml. of 2·0 M-tris–HCl buffer, pH 7·2, 0·40 ml. of root extract; total volume 0·60 ml. Temp., 37°. □, Uridine; △, UMP; ▲, UTP; ○, UDPG; ●, UDP.

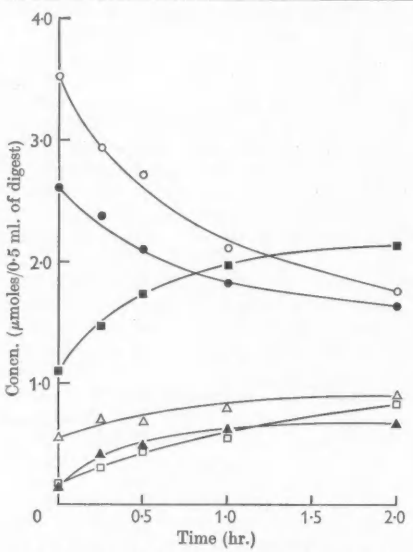


Fig. 4. Synthesis of UTP from ATP plus UDP by an extract of sugar-beet root. Reaction mixtures contained: 3·52 μmoles of ATP (plus 1·10 μmoles of ADP and 0·17 μmole of AMP as impurities), 2·60 μmoles of UDP (plus 0·55 μmole of UMP and 0·14 μmole of UTP as impurities), 0·02 ml. of 2·0 M-tris–HCl buffer, pH 7·2, 0·01 ml. of root extract; total volume 0·50 ml. Temp. 37°. □, AMP; ■, ADP; ○, ATP; ●, UDP; △, UMP; ▲, UTP.

thesis of UTP, further experiments showed that this nucleotide could be replaced in the UDPG synthesis (6) by similar concentrations of ATP plus UDP and that the rate of formation of UDPG was not affected. No UDPG was found if any one of the three substrates, glucose 1-phosphate, ATP or UDP, was omitted from the digests. The overall reaction is presumably catalysed by nucleoside diphosphokinase and UDPG pyrophosphorylase:

$$\text{G1-P} + \text{UDP} + \text{ATP} \rightleftharpoons \text{G1-P} + \text{UTP} + \text{ADP} \rightleftharpoons \text{UDPG} + \text{P-P} + \text{ADP} \quad (8)$$

Glucose 1-phosphate could not be replaced by glucose in the UDPG synthesis. Nor did incubation of glucose and ATP with root extract yield any glucose 6-phosphate, indicating low or no hexokinase activity. Glucose 6-phosphate was, however, shown to be present in the root and the amount extracted by the barium technique of LePage (1957) indicated a minimum concentration of 10 $\mu$moles/100 g. of root.

*Synthesis of sucrose from fructose, glucose 1-phosphate, adenosine triphosphate and uridine diphosphate.* Since UDPG could be synthesized from glucose 1-phosphate, ATP and UDP, the overall synthesis of sucrose by combination of reactions 2 and 8 was attempted by incubation of a digest containing the root extract with fructose, glucose 1-phosphate, ATP and UDP.

The digest, containing 0·02 ml. of tris-hydrochloride buffer, pH 7·2, 6·5 $\mu$moles of fructose, 6·5 $\mu$moles of glucose 1-phosphate, 1·4 $\mu$moles of UDP, 1·2 $\mu$moles of ATP and 0·3 ml. of root extract in a total volume of 0·6 ml., after incubation at 37° for 3 hr. yielded a detectable sucrose spot on paper chromatograms. A sucrose spot was not present on chromatograms of blank digests from which any single component was omitted.

## DISCUSSION

UDPG is shown to be present in all sections of the sugar-beet plant and it is suggested that sucrose may be synthesized, and UDPG regenerated, in the root by the overall reaction (9):

$$\text{ATP} + \text{UDP} \rightleftharpoons \text{UTP} + \text{ADP} \quad (7)$$

$$\text{UTP} + \text{G1-P} \rightleftharpoons \text{UDPG} + \text{P-P} \quad (6)$$

$$\text{Fructose} + \text{UDPG} \rightleftharpoons \text{sucrose} + \text{UDP} \quad (2)$$

$$\text{Fructose} + \text{G1-P} + \text{ATP} \rightleftharpoons \text{sucrose} + \text{ADP} + \text{P-P} \quad (9)$$

Enzymes capable of effecting reactions (7), (6) and (2) are demonstrably present in the root and in summation the synthesis requires only a source of fructose and ATP, which are readily available, and of glucose 1-phosphate. The root is also shown to contain phosphoglucomutase and glucose 6-phosphate, which can provide a source of glucose 1-phosphate. It therefore appears that a complete mechanism for sucrose synthesis occurs in the sugar-beet root.

The conclusions about sucrose synthesis by sugar beet have been conflicting. It has been suggested (Kursanov & Pavlinova, 1952; Pavlinova, 1954; Kursanov, 1954) that sucrose synthesis does not occur in the mature root and that the sucrose is accumulated in the root solely by transfer of sucrose or sucrose phosphate from the blade. This passive role was allocated to the root because sucrose synthesis was readily detected when monosaccharides were introduced into leaf disks but not when the same technique was applied to mature roots.

The synthesis of sucrose from UDPG and fructose reported here was achieved on many occasions and with extracts of roots that were prepared throughout the growing period of the plant. It seems therefore unlikely that failure by others to achieve the synthesis could be due to the fact that they used the plant only at a particular stage of its growth. Differences in technique in preparing the extracts may well be the main cause of confusion. For instance, Cardini *et al.* (1955) dialysed root extracts against water and it is possible to show that UDPG is degraded quite rapidly by extracts prepared in this way. Degradation is slowed down considerably when dialysis is carried out, as described in this paper, against phosphate buffer.

Rorem *et al.* (1960) obtained enzyme extracts from blade which catalysed sucrose synthesis from fructose and UDPG and also sucrose phosphate synthesis from fructose 6-phosphate and UDPG, and Burma & Mortimer (1956) demonstrated sucrose phosphate synthesis from fructose 6-phosphate and UDPG but found no sucrose if fructose 6-phosphate was replaced by fructose. In the present investigations, no sucrose synthesis was detected with blade extracts and neither sucrose nor sucrose phosphate was produced in extracts incubated with fructose 6-phosphate.

It would appear that no particular route of synthesis should be eliminated solely on the basis of negative results.

The blade extracts of Rorem *et al.* (1960) were prepared by a slightly different technique and partial elimination of invertase activity was attempted both during extraction and by incubation of digests at pH 8·7. A high invertase activity in the digest would, however, be required to prevent qualitative detection of sucrose synthesis and, in the present work, addition to the root extract of an equal volume of blade extract did not produce a sensible inhibiting effect on the sucrose synthesis.

The difficulties of interpreting results obtained from extracts of tissue are illustrated by the

apparent distribution of hexokinase and phosphoglucomutase. Kursanov, Pavlinova & Afanasieva (1959) reported the former to be present and the latter absent in petioles and the reverse picture is presented here for root extracts. These observations do not, however, preclude the possibility that both enzymes are present in the root and the petiole of the intact plant. Similarly the present negative findings with blade extract are not to be interpreted as a suggestion that UDPG is not involved in sucrose synthesis in beet leaves.

It is considered that a mechanism for the synthesis of sucrose occurs both in the root and in the blade of the sugar beet and it may be important to consider both potential sources in assessing the overall production of sucrose by the plant.

## SUMMARY

1. The root of the sugar beet was found to contain a complete mechanism for the synthesis of sucrose.

2. Uridine diphosphate glucose, uridine diphosphate, adenosine triphosphate and adenosine diphosphate were present at similar concentrations in the blade, petiole, crown and root of sugar beet.

3. Enzyme extracts of the root or crown effected synthesis of sucrose from uridine diphosphate glucose and fructose.

4. The extracts were also shown to possess uridine diphosphate glucose-pyrophosphorylase and nucleoside-diphosphokinase activity, indicating that the mechanism was present in the root for regeneration of uridine diphosphate glucose.

5. The successive syntheses by the above-mentioned enzymes of uridine triphosphate, uridine diphosphate glucose and sucrose were demonstrated on incubation of a single digest containing adenosine triphosphate, uridine diphosphate, glucose 1-phosphate and fructose. The root also contained phosphoglucomutase and glucose 6-phosphate, which can provide a source of glucose 1-phosphate.

6. The kinetics of the synthesis of the sucrose and the interconversion of the nucleotides were examined. The reactions were complicated by nucleotide-pyrophosphatase, nucleotidase, myokinase and apyrase activity.

7. Contrary to previous views, it is concluded that the root of the sugar beet, as well as the leaf, may play a significant role in the synthesis of sucrose by the plant.

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# The Metabolism of $C_2$ Compounds in Micro-Organisms

## 7. PREPARATION AND PROPERTIES OF CRYSTALLINE TARTRONIC SEMIALDEHYDE REDUCTASE*

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The biosynthesis of cell materials by *Pseudomonas* spp. growing on glycollate (Kornberg & Gotto, 1959, 1961), on glycine (Callely & Dagley, 1959), on oxalate (Quayle & Keech, 1959) or on other substrates catabolized to glyoxylate (Gray, Gerhart & Brooke, 1959) has been shown to be effected by a sequence of reactions in which, initially, glyoxylate is converted into glycerate. This transformation is achieved by the successive action of two adaptively formed enzymes: glyoxylate carboligase (Krakow & Barkulis, 1956; Krakow, Hayashi & Barkulis, 1959; Krakow, Barkulis & Hayashi, 1961), which catalyses the condensative decarboxylation of glyoxylate to tartronic semialdehyde:

$$2CHO\cdot CO_2H \xrightarrow[\text{thiamine pyrophosphate}]{Mg^{2+}\text{ ions}} CO_2 + CHO\cdot CH(OH)\cdot CO_2H \quad (1)$$

and tartronic semialdehyde reductase (Gotto & Kornberg, 1961*a*), which catalyses the reduction of the tartronic semialdehyde thus formed to glycerate:

$$CHO\cdot CH(OH)\cdot CO_2H + DPNH\ (\text{or } TPNH) + H^+ \rightarrow HO\cdot CH_2\cdot CH(OH)\cdot CO_2H + DPN^+\ (\text{or } TPN^+) \quad (2)$$

It is the main purpose of this paper to describe a method for the preparation of this latter enzyme in a crystalline state and to discuss some of the properties of the enzyme thus prepared. Preliminary reports of portions of this work have been published (Gotto & Kornberg, 1961*a*, *b*).

## MATERIALS AND METHODS

*Enzymic preparation of tartronic semialdehyde.* Tartronic semialdehyde was prepared enzymically by the treatment of glyoxylate with glyoxylate carboligase. The main compartment of a Warburg manometer flask contained: 50 $\mu$moles of potassium phosphate, pH 6·5; 0·5 $\mu$mole of thiamine pyrophosphate; 5 $\mu$moles of $MgCl_2$; 0·05 ml. of purified glyoxylate carboligase (see below). Side arm 1 contained 80 $\mu$moles of sodium glyoxylate and side arm 2 contained 0·20 ml. of 20% perchloric acid. The flasks were equilibrated at 30° under $N_2$ for 10–15 min., after which time the glyoxylate was added from the first side arm. When evolution of $CO_2$ ceased (approx. 10 min.), the perchloric acid was added from the second side arm, the cup was detached from its manometer and the contents of the manometer flask were centrifuged immediately at 2°. (Continued shaking of the flask, after addition of the perchloric acid, was found to result in considerable decarboxylation of the tartronic semialdehyde.) After centrifuging at 20 000*g* for 2 min., the pH was adjusted to 7·0–7·5 by dropwise addition of 2 N-KOH, and the potassium perchlorate was removed by centrifuging at 2° for 15 min. The concentration of tartronic semialdehyde in the neutralized solution was calculated from the total change in $E_{340\,m\mu}$ when samples were incubated with the purified tartronic semialdehyde reductase (see below) in the presence of an excess of reduced DPN (DPNH). The yields obtained varied between 30 and 60% of the amounts expected from the measured quantities of $CO_2$ evolved from glyoxylate. Since the product is rather unstable, the tartronic semialdehyde solutions are best prepared freshly each day.

*Chemical preparation of tartronic semialdehyde.* Authentic tartronic semialdehyde was prepared by Professor D. B. Sprinson from the reductive cleavage of the benzyl *cyclo*acetal derivative (Fischer, Baer & Nidecker, 1937). Since this material became available only after most of the work described in this paper had been completed, the enzymically prepared tartronic semialdehyde was used for most experiments. However, no significant difference was detected between the properties of the enzymic and chemical products.

*Potassium* D-*glycerate.* The quinine salt of D-glycerate, which was a gift from Dr P. W. Kent, was treated with Dowex 50 resin in the $H^+$ form. The solution thus obtained was titrated with 0·2 N-KOH to pH 7: the glycerate content was calculated from the amount of alkali required. High-voltage paper electrophoresis of a sample of the glycerate solution, in 0·5 M-pyridine/0·5 M-acetic acid adjusted to pH 4·0 (Gross, 1959), showed glyceric acid to be the sole acid constituent detectable.

*Sodium* [1-$^{14}$C]*glyoxylate.* Calcium [1-$^{14}$C]glyoxylate was purchased from The Radiochemical Centre, Amersham, Bucks., and was converted into the sodium salt by treatment with Dowex 50 resin in the $Na^+$ form. It was purified, in collaboration with Dr I. Zelitch, as follows.

The [1-$^{14}$C]glyoxylate solution was loaded on to a chromatography column (0·7 cm. diam.), filled to a height of

* Part 6: Kornberg & Gotto (1961).

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6 cm. with Dowex 1 (X8) resin in the acetate form. The material was washed with water and the [1-$^{14}$C]glyoxylic acid was eluted with 4 M-acetic acid. Those fractions of the eluate containing [1-$^{14}$C]glyoxylic acid, as determined by the method of Friedemann & Haugen (1943) and by radioassay, were combined and the acetic acid was removed by freeze-drying. The [1-$^{14}$C]glyoxylic acid was neutralized with 0·2 M-KOH: it was found to be 100% pure by chromatography and radioassay of the 2:4-dinitrophenylhydrazone (El Hawary & Thompson, 1953).

*Lithium hydroxypyruvate.* The lithium hydroxypyruvate used was a gift from Professor F. Dickens, F.R.S., and Mr D. H. Williamson.

*Malonic semialdehyde.* Ethyl $\beta\beta$-diethoxypropionate, which was a gift from Professor M. J. Coon, was converted into malonic semialdehyde as described by Robinson & Coon (1961).

*Mesoxalic semialdehyde.* Dihydroxyfumaric acid was converted into mesoxalic semialdehyde by the procedure of Fenton (1905).

*Reductone.* Reductone was a gift from Dr J. G. Morris.

*Other reagents used.* Reduced and oxidized pyridine nucleotides, crystalline lactic dehydrogenase, phospho*enol*pyruvate and 3-phosphoglycerate were purchased from C. F. Boehringer und Soehne (Mannheim, Germany); $\beta$-hydroxypropionic acid was from the California Corp. for Biochemical Research (Los Angeles, U.S.A.); thiamine pyrophosphate was from the Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.); 2:6-dichlorophenol-indophenol, DL-glyceraldehyde, dihydroxyfumaric acid and glyoxylic acid were from British Drug Houses Ltd. (Poole, Dorset); protamine sulphate ('ex herring') and L-malic acid were from L. Light and Co. Ltd. (Colnbrook, Bucks.). Oxaloacetic acid, sodium pyruvate and L-lactate were gifts from Professor Sir Hans Krebs, F.R.S. All other reagents used were of the highest purity commercially available.

### *Preparation of tartronic semialdehyde reductase*

Mass cultures of glycollate-grown *Pseudomonas ovalis* Chester (Dixon, Kornberg & Lund, 1960) were generously supplied by Mr R. Elsworth (Ministry of Aviation, Microbiological Research Establishment, Porton, Wilts.). Tartronic semialdehyde reductase and other enzymes required were prepared from the frozen packed cells as follows.

*Step* 1: *preparation of ultrasonic extracts.* Frozen cells (80 g.) were thawed and diluted to 200 ml. with 5 mM-sodium potassium phosphate, pH 7·0 (prepared by mixing 61·1 vol. of 5 mM-$Na_2HPO_4$ with 38·9 vol. of 5 mM-$KH_2PO_4$). The suspension was subjected (in 20 ml. batches) for 5 min. to the output of a 600 w Mullard magnetostrictor oscillator operating at 3·5 A. After this treatment, the batches were combined and centrifuged for 1 hr. at 35 000*g* in a MSE Speed 17 centrifuge at 2°. The precipitate was discarded. All further operations were carried out at 0–2°, the enzymes being assayed at each step as described below.

*Steps* 2–3: *treatment with protamine sulphate and alumina $C_\gamma$-gel.* After centrifuging, 2% (w/v) protamine sulphate solution (1·5 mg. of protamine sulphate for each 10 mg. of soluble protein) was added slowly to the supernatant solution. The precipitate was removed by centrifuging for 30 min. at 35 000*g*. To each 100 ml. of supernatant solution was added 20 ml. of alumina $C_\gamma$-gel [30 mg. dry wt./ml.; prepared as described by Colowick (1955)]. The precipitate was removed by centrifuging and discarded.

*Step* 4: *fractionation with ammonium sulphate.* To each 100 ml. of supernatant solution was added 24·3 g. of solid $(NH_4)_2SO_4$ (40% saturation). The precipitate was collected by centrifuging and was discarded. To each 100 ml. of the supernatant solution was added 24·5 g. of solid $(NH_4)_2SO_4$ (75% saturation); the precipitate was collected by centrifuging and was dissolved in 15–20 ml. of 5 mM-phosphate buffer, pH 7·0, containing 1 mM-mercaptoethanol. This material was dialysed at 2° against 2 l. of 5 mM-sodium potassium phosphate, pH 7·0, and 1 mM-mercaptoethanol. This buffer mixture was replaced by 2 l. of fresh buffer mixture after 9 hr. and dialysis was continued for a further 9 hr. The small quantity of material precipitated during dialysis was removed by centrifuging.

*Step* 5: *column chromatography.* A batch of 7 g. of diethylaminoethylcellulose (DEAE-cellulose; Whatman DE 50) was suspended in 500 ml. of 5 mM-sodium potassium phosphate buffer, pH 7·0, and the pH of the suspension was re-adjusted to 7·0 by the addition of 2 N-HCl. The cellulose was washed several times with 5 mM-phosphate and particles which did not sediment were removed by decantation. The slurry was then poured into a chromatographic column (2 cm. × 30 cm.), the lower end of which was closed with glass wool. The column was equilibrated at 2° by allowing 1 l. of 5 mM-sodium potassium phosphate, pH 7·0, to run through it. The dialysed solution was applied to the DEAE-cellulose column at a rate just sufficient to keep the top of the column moist: by this means it was possible to adsorb the material in a narrow band at the top of the column. A linear gradient of KCl was now applied to the column by allowing 400 ml. of a solution containing 5 mM-sodium potassium phosphate, pH 7·0, 1 mM-mercaptoethanol and 50 mM-KCl to mix, with constant stirring, with 400 ml. of a solution containing 5 mM-sodium potassium phosphate, pH 7·0, 1 mM-mercaptoethanol and 450 mM-KCl: this mixture was allowed to flow through the column at 40–50 ml./hr. Fractions, each containing approx. 8 ml. (95 drops), were collected with a Locarte automatic fraction collector.

The hold-up volume, i.e. the material which was not adsorbed on the column, was rich in DPNH-dehydrogenase activity, but was devoid of tartronic semialdehyde reductase. It was precipitated by the addition of solid $(NH_4)_2SO_4$ to 75% of saturation (516 mg./ml.). The precipitated material was dissolved in 5 mM-phosphate, pH 7·0 and stored at 2°. Samples, suitably diluted in buffer, were used as the source of DPNH-dehydrogenase in the procedures described below.

Tartronic semialdehyde reductase was eluted from the column between 110 and 180 mM-KCl, the peak of activity appearing at 130 mM-KCl (Fig. 1). Those fractions containing the enzyme at specific activities (see below) greater than 30 were combined and the enzyme was precipitated by the addition of $(NH_4)_2SO_4$ to a final concentration of 75% of saturation.

Glyoxylate carboligase (see below), which was eluted between 200 and 250 mM-KCl, was similarly precipitated by the addition of solid $(NH_4)_2SO_4$ to 75% of saturation. This material retained full enzymic activity for several months when stored at 2° in the presence of crystalline bovine serum albumin and 5 mM-sodium potassium phos-

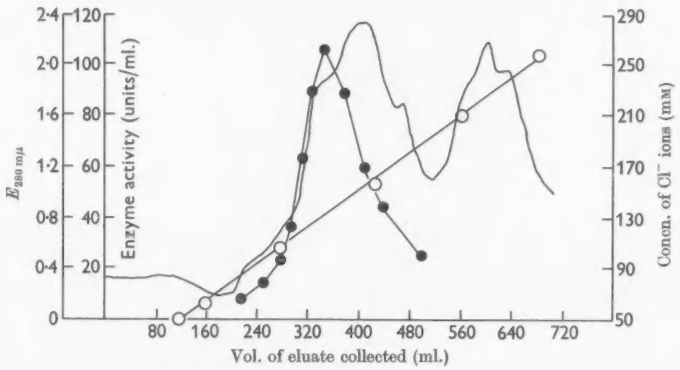


Fig. 1. Elution of tartronic semialdehyde from DEAE-cellulose. For conditions see text. The continuous line represents the protein concentration, measured as $E_{280\,m\mu}$. ●, Tartronic semialdehyde-reductase activity; ○, gradient of chloride concentration.

phate, pH 7·5, containing 1 mM-$MgCl_2$ and 0·1 mM-thiamine pyrophosphate.

*Steps 6–7: crystallization.* The $(NH_4)_2SO_4$ precipitate of tartronic semialdehyde reductase was collected by centrifuging and was dissolved in 3–4 ml. of 5 mM-phosphate, pH 7·0. Any material which did not dissolve at this point was removed by centrifuging. The supernatant solution was brought to first turbidity (approx. 40% of saturation) by the slow addition of solid $(NH_4)_2SO_4$. The material was allowed to warm slowly to room temperature (21°), during which time crystallization began, and was maintained at this temperature for 1 hr. and at 0° for a further period of 4–5 hr. The crystalline precipitate was collected by centrifuging, dissolved in the minimal volume of 5 mM-sodium potassium phosphate buffer, pH 7·0, and recrystallized three times with $(NH_4)_2SO_4$ as described above. These successive recrystallizations were not accompanied by any significant change in the specific activity of the enzyme. The crystals (Fig. 2) appeared to have the shape of two pyramids fused at their bases. The material sedimented in the ultracentrifuge as a single symmetrical peak (Fig. 3).

The simple and reproducible purification procedure is summarized in Table 1.

D-*Glyceric dehydrogenase.* D-Glyceric dehydrogenase (Stafford, Magaldi & Vennesland, 1954; Holzer & Holldorf, 1957) was prepared in collaboration with Dr I. Zelitch. Tobacco leaves (four), Havana variety, weighing 35 g. after removal of the midribs, were ground with a little sand and 35 ml. of water, 310 mg. of cysteine–HCl and 250 mg. of $KHCO_3$. The extract was filtered through a double layer of cheese cloth and the filtrate was centrifuged twice at 20 000*g* for 20 and 10 min. periods respectively. To the supernatant solution, solid $(NH_4)_2SO_4$ was added to 40% saturation. The precipitate was dissolved in 4·5 ml. of 10 mM-potassium phosphate, pH 7·0, containing 5 mM-cysteine–HCl, and was stored frozen at −12°.

### *Assay of enzymes*

All spectrophotometric assays were performed in silica cells, 1·5 ml. vol., 1 cm. light-path, in a Cary model 14 recording spectrophotometer at 23±1°.

*Reduced diphosphopyridine nucleotide dehydrogenase.* This was estimated by measurement of the rate of change of $E_{600\,m\mu}$, consequent upon the enzymic reduction of 2:6-dichlorophenol-indophenol in the presence of the enzyme and DPNH. The complete system contained, in 1 ml.: 0·4 ml. of 0·01% (w/v) 2:6-dichlorophenol-indophenol; 0·05 ml. of 0·1 M-KCN adjusted to pH 8·5; 0·1 ml. of 1 M-disodium hydrogen phosphate, pH 8·5; appropriate amounts of enzyme. $E_{600\,m\mu}$ of this solution was measured for 1–2 min., after which time the reaction was started by addition of 0·1 ml. of 10 mM-DPNH. One unit of enzyme was defined as $\Delta E_{600\,m\mu} = -1{\cdot}0$ unit/min.

*Glyoxylate carboligase.* This was assayed manometrically as described by Kornberg & Gotto (1961) or spectrophotometrically by measurement of the rate of oxidation of DPNH when this enzyme was incubated with glyoxylate, in the presence of its required cofactors $Mg^{2+}$ ions and thiamine pyrophosphate, and an excess of tartronic semialdehyde reductase. The complete system contained, in 1 ml.: 0·1 ml. of 1 M-potassium phosphate, pH 7·5; 0·05 ml. of 0·1 M-$MgCl_2$; 0·05 ml. of 10 mM-thiamine pyrophosphate; 0·02 ml. of 10 mM-DPNH; an excess (determined as described later) of crystalline tartronic semialdehyde reductase. $E_{340\,m\mu}$ was recorded for 1–2 min., after which time the reaction was started by addition of 0·03 ml. of 0·2 M-sodium glyoxylate. One unit of enzyme is defined as that quantity catalysing the oxidation of 1 $\mu$mole of DPNH/min., thus giving $\Delta E_{340\,m\mu} = -6{\cdot}28$ units/min.

*Tartronic semialdehyde reductase.* This was assayed by measurement (*a*) of the rate of oxidation of DPNH in the presence of the enzyme and tartronic semialdehyde, or (*b*) of the rate of reduction of 2:6-dichlorophenol-indophenol in the presence of the enzyme, D-glycerate, $DPN^+$ and excess of DPNH-dehydrogenase.

(*a*) In the former assay, the complete system contained, in 1 ml.: 0·1 ml. of 1 M-disodium hydrogen phosphate, pH 8·5; 0·02 ml. of 10 mM-DPNH; an appropriate amount of enzyme. $E_{340\,m\mu}$ was recorded for 1–2 min., after which time the reaction was started by the addition of 0·05 ml. of 10 mM-tartronic semialdehyde. One unit of enzyme is defined as that quantity which catalyses the oxidation of 1 $\mu$mole of DPNH/min. and thus gives $\Delta E_{340\,m\mu} = -6{\cdot}28$ units/min.

(*b*) In the latter assay, the complete system contained, in 1 ml.: 0·4 ml. of 0·01% (w/v) 2:6-dichlorophenol-indophenol; 0·1 ml. of 1 M-disodium hydrogen phosphate, pH 8·5; 0·05 ml. of 0·1 M-KCN; 0·02 ml. of DPNH-dehydrogenase; 0·1 ml. of 10 mM-$DPN^+$; an appropriate amount of the enzyme. $E_{\text{600 m}\mu}$ was recorded for 1–2 min., after which time the reaction was started by the addition of 0·05 ml. of 70 mM-potassium D-glycerate. One unit of enzyme in this assay is defined as that quantity which catalyses $\Delta E_{\text{600 m}\mu} = -1{\cdot}0$ unit/min. This quantity is equivalent to 0·39 unit of enzyme determined under the conditions of the former assay (*a*).

*Specific activity.* A specific activity of 1 is defined as 1 unit of enzyme/mg. of soluble protein.

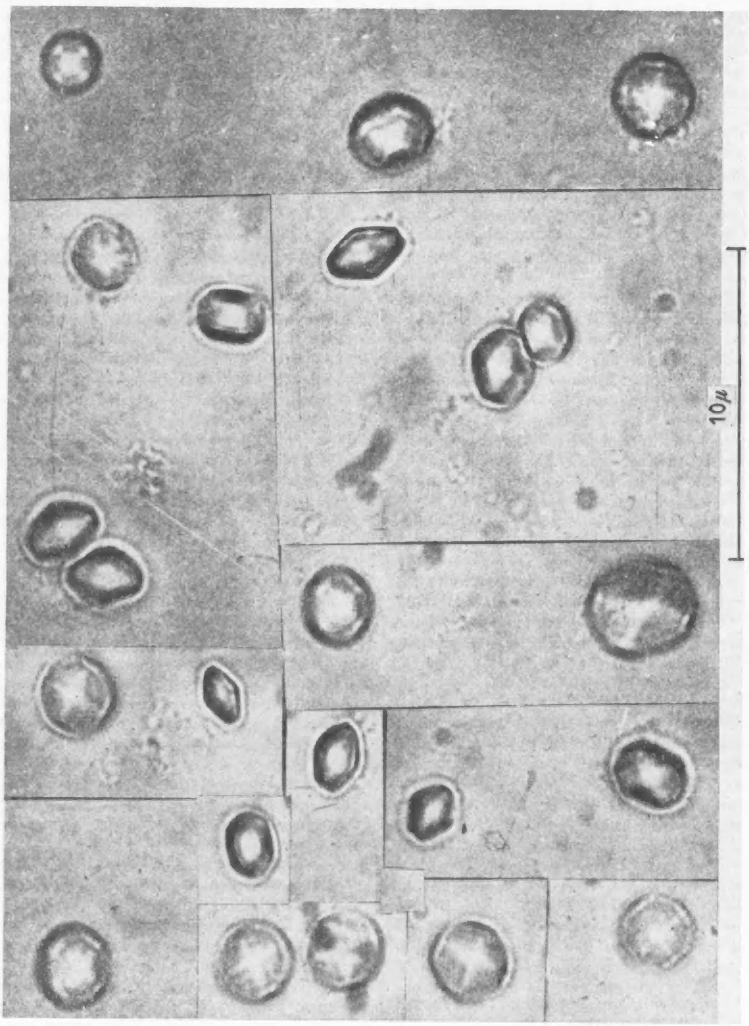


Fig. 2. Crystals of tartronic semialdehyde reductase; composite photograph to show crystalline structure (magnification ×3380). Photograph by Dr R. Barer (Department of Human Anatomy, University of Oxford).

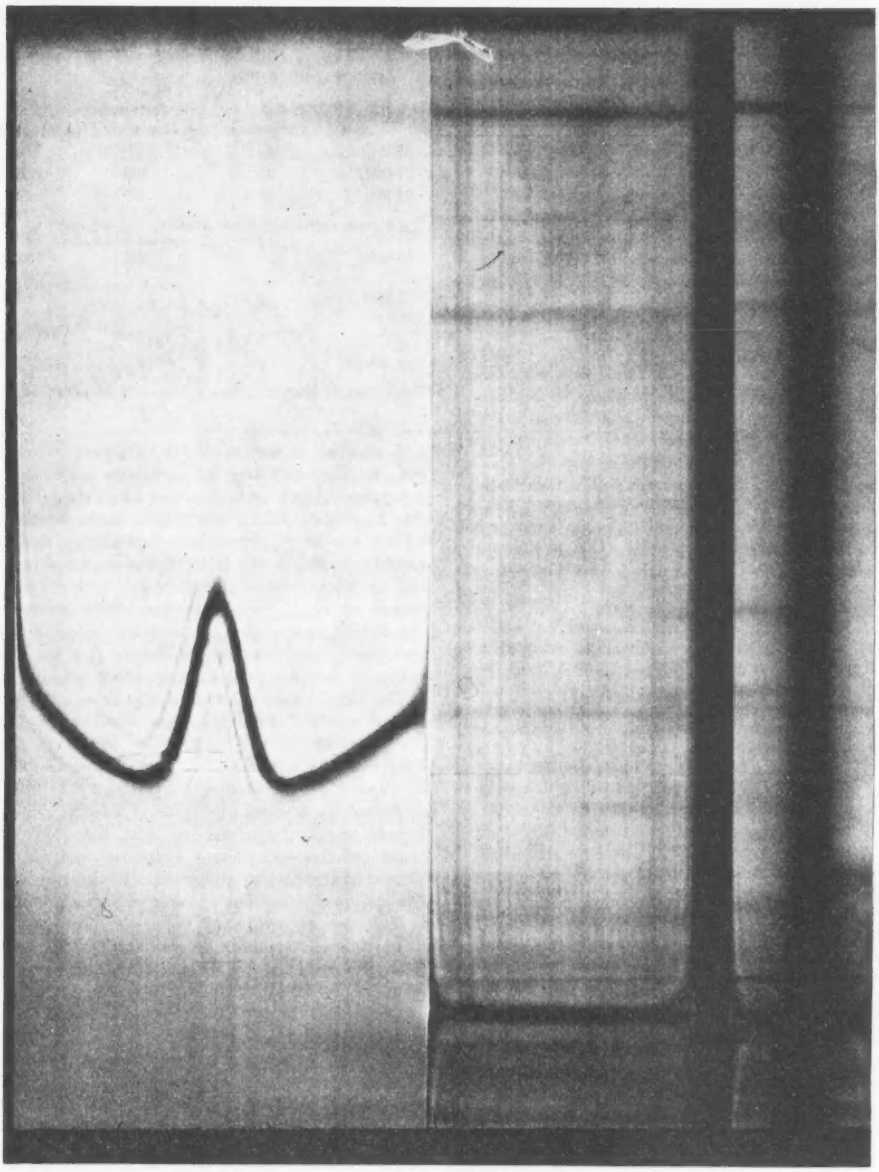

Fig. 3. Sedimentation pattern of crystalline tartronic semialdehyde reductase. The enzyme was dissolved in 0·1 M-sodium potassium phosphate buffer, pH 7·4. Sedimentation was from right to left at phase-plate angle 35° and rotor temperature 3·2°. The photograph was taken after 64 min. at 59 780 rev./min.

Table 1. *Summary of purification of tartronic semialdehyde reductase*

The enzyme was assayed by measurement of the rate of decrease of $E_{600\,m\mu}$. The complete system contained, in 1 ml.: 100 $\mu$moles of disodium hydrogen phosphate, pH 8·5; 5 $\mu$moles of KCN; 3·5 $\mu$moles of D-glycerate; 0·4 ml. of 0·01% 2:6-dichlorophenol-indophenol; 0·5 $\mu$mole of $DPN^+$; 0·02 ml. of DPNH-dehydrogenase and enzyme. One unit of enzyme is defined as that quantity catalysing $\Delta E_{600\,m\mu} = -1$ unit/min.

| Step | Vol. of soln. (ml.) | Units | Concn. of protein (mg./ml.) | Specific activity (units/mg. of protein) | Recovery (%) |
|---|---|---|---|---|---|
| 1. Sonic extract | 180 | 27 000 | 65 | 2·3 | 100 |
| 2. Supernatant from protamine sulphate treatment | 230 | 24 000 | 21·5 | 4·8 | 89 |
| 3. Supernatant from $C_\gamma$-gel adsorption | 290 | 24 000 | 13·5 | 6·3 | 89 |
| 4. Ammonium sulphate precipitate, 40–75% saturated | 20 | 15 000 | 100 | 7·4 | 55 |
| 5. Pooled selected fractions from DEAE-cellulose column | 100 | 7 000 | 1·82 | 37 | 26 |
| 6. Crystals, crop I | 1 | 2 100 | 5·5 | 385 | 7·8 |
| 7. Crop I, recrystallized three times | 2 | 2 000 | 2·5 | 410* | 7·4 |

* This corresponds to the oxidation of 160 $\mu$moles of DPNH/min./mg. of protein in the presence of excess of tartronic semialdehyde.

### *Other methods*

*Estimation of protein.* The protein content of crude sonic extracts, and of the material obtained in steps 1–4 of the purification procedure, was estimated by the biuret method of Gornall, Bardawill & David (1949). The protein content of materials obtained in steps 5–7 of the purification procedure was estimated by the spectrophotometric method of Warburg & Christian (1941).

*Estimation of chloride.* The chloride content of materials eluted from DEAE-cellulose columns was determined by the method of West & Coll (1957).

*Chromatographic and radioautographic procedures.* The methods used for chromatography and radioautography have been previously described (Kornberg, 1958; Kornberg & Gotto, 1961).

*Thermodynamic symbols.* The symbols used are based on those given in the *Report of the Royal Society Committee on Symbols* (1951) (cf. Burton & Krebs, 1953).

## RESULTS

*Enzymic reduction of tartronic semialdehyde.* When tartronic semialdehyde, prepared enzymically or chemically, was added to cuvettes containing tartronic semialdehyde reductase and DPNH, $E_{340\,m\mu}$ of the solution decreased rapidly. This reaction occurred only when both enzyme and substrate were present, and did not take place if the enzyme was boiled before incubation. In the presence of excess of DPNH and enzyme, the total decrease of $E$ was stoicheiometrically related to the quantity of tartronic semialdehyde added (reaction 2). In the presence of excess of tartronic semialdehyde, the initial rates of oxidation of DPNH were proportional to the quantities of crystalline enzyme added over the range at which such rates could be conveniently measured (0·1–1·0 $\mu$g. of enzyme protein/ml.).

A similar oxidation of DPNH, proportional in rate to the quantity of tartronic semialdehyde reductase added, was observed when the substrate was generated during the course of the incubation by an excess of glyoxylate carboligase and glyoxylate (reaction 1). DPNH was oxidized only in the complete system (reactions 1 and 2); no decrease in $E_{340\,m\mu}$ was detected when glyoxylate, glyoxylate carboligase or tartronic semialdehyde reductase was omitted or when the combined enzymes were boiled before incubation (Fig. 4).

The net formation of glycerate from glyoxylate in this coupled enzyme system was demonstrated by using sodium [1-$^{14}$C]glyoxylate as the substrate. Under these conditions, half the isotope added was evolved as $^{14}CO_2$ (Kornberg & Gotto, 1961) and half appeared in labelled glycerate (Table 2). No such formation of [$^{14}$C]glycerate was detected when either of the constituent enzymes was omitted. This confirmed that glycerate was the product of the enzymic reduction of tartronic semialdehyde.

*Enzymic oxidation of glycerate.* The enzymic formation of tartronic semialdehyde and DPNH from glycerate and $DPN^+$ (reaction 2, from right to left, i.e. reaction 2*a* below) proved difficult to demonstrate directly as the equilibrium of the system greatly favoured glycerate formation (see later), but was readily demonstrated when the DPNH formed was reconverted into DPN. This was done in two ways. In the first, the oxidation of glycerate, catalysed by crystalline tartronic semialdehyde reductase, was coupled to the reduction of pyruvate, catalysed by crystalline lactic dehydrogenase (reaction 3) in the presence of $DPN^+$. The overall removal of pyruvate (reaction 4) depended on the presence of both enzymes, glycerate and $DPN^+$ (Table 3).

$$\text{Glycerate} + \text{DPN}^+ \rightarrow \text{tartronic semialdehyde} + \text{DPNH} + \text{H}^+ \quad (2a)$$

$$\text{Pyruvate} + \text{DPNH} + \text{H}^+ \rightarrow \text{lactate} + \text{DPN}^+ \quad (3)$$

$$\text{Sum: Glycerate} + \text{pyruvate} \rightarrow \text{tartronic semialdehyde} + \text{lactate} \quad (4)$$

In the second method, which also served as the basis of the convenient assay procedure (*b*) (see Materials and Methods section) for tartronic semialdehyde reductase, the DPNH formed from $DPN^+$ concomitantly with glycerate oxidation was reconverted into $DPN^+$ in the presence of DPNH-dehydrogenase and 2:6-dichlorophenol-indophenol as electron acceptor. Although the relatively crude enzyme obtained in steps 1–4 (Table 1) of the purification procedure catalysed the reduction of 2:6-dichlorophenol-indophenol in the presence of glycerate and $DPN^+$, and without addition of DPNH-dehydrogenase, the material obtained after DEAE-cellulose column chromatography (step 5, Table 1) did not catalyse the reduction of the dye unless DPNH-dehydrogenase was also present. The enzymic oxidation of glycerate in this system again required the presence of the enzyme, glycerate, catalytic amounts of $DPN^+$, DPNH-dehydrogenase and the electron acceptor: no glycerate oxidation was observed if any one of these constituents was omitted (Fig. 5). The initial rate of dye reduction was proportional to the amounts of enzyme added over the range at which such rates could be conveniently measured (0·1–1·0 $\mu$g. of protein/ml.). The material formed in the complete system formed a bis derivative with 2:4-dinitrophenylhydrazine which behaved identically with the derivative prepared from the enzymically or chemically prepared tartronic semialdehyde.

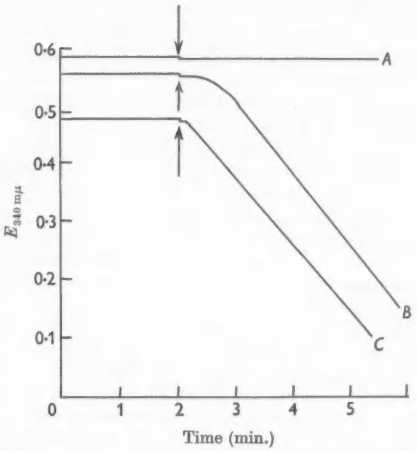


Fig. 4. Formation of glycerate from glyoxylate by purified enzymes. The complete system contained, in 1·0 ml.: 50 $\mu$moles of potassium phosphate, pH 7·5; 5 $\mu$moles of $MgCl_2$; 0·5 $\mu$mole of thiamine pyrophosphate; 0·1 $\mu$mole of DPNH; purified glyoxylate carboligase (0·25 mg.); tartronic semialdehyde reductase (0·05 mg.). Glycerate formation was measured as the rate of decrease in $E_{340\,m\mu}$. The reactions were initiated by the addition, at the times indicated by arrows, of glyoxylate carboligase to *A* and *B*, and of tartronic semialdehyde reductase to *C*. In curve *A*, glyoxylate was also omitted from the reaction mixture. (Taken from tracings made with a Cary recording spectrophotometer.)

*Equilibrium of the reaction.* The apparent and thermodynamic equilibrium constants, $K$ and $K_H$ respectively, for reaction 2*a* were calculated from the equations:

$$K = \frac{[\text{tartronic semialdehyde}^-]\,[\text{DPNH}]}{[\text{glycerate}^-]\,[\text{DPN}^+]}$$

and

$$K_H = \frac{[\text{tartronic semialdehyde}^-]\,[\text{DPNH}]\,[\text{H}^+]}{[\text{glycerate}^-]\,[\text{DPN}^+]}$$

The equilibrium concentrations were measured by adding various amounts of glycerate to $DPN^+$ and crystalline tartronic semialdehyde reductase, and

Table 2. *Conversion of* [1-$^{14}$C]*glyoxylate into* [$^{14}$C]*glycerate by purified enzymes*

The complete system contained, in a total volume of 3·0 ml.: 100 $\mu$moles of potassium phosphate, pH 7·5; 10 $\mu$moles of magnesium chloride; 0·5 $\mu$mole of thiamine pyrophosphate; 1 $\mu$mole of DPNH; 0·10 ml. of a solution of sodium [1-$^{14}$C]glyoxylate (giving $1 \times 10^5$ counts/min. under the conditions of radioassay used); 0·2 mg. of glyoxylate carboligase; 0·3 mg. of crystalline tartronic semialdehyde reductase. After incubation at 23° for 30 min., 0·30 ml. was pipetted into 0·10 ml. of saturated 2:4-dinitrophenylhydrazine in 2N-HCl, and the remainder was poured into 4·0 ml. of ethanol. These two fractions were analysed by chromatography and their radioactivity was measured as described by Kornberg (1958) and Kornberg & Gotto (1961).

| | $10^{-3} \times$ Radioactivity (counts/min.) | | | |
|---|---|---|---|---|
| | Glyoxylate | | Glycerate | |
| Reaction mixture | (Initial) | (Final) | (Initial) | (Final) |
| Complete system | 100 | 0·04 | 0 | 49·7 |
| Glyoxylate carboligase omitted | 100 | 100 | 0 | 0 |
| Tartronic semialdehyde reductase omitted | 100 | 98·6 | 0 | 0·43 |

following $E_{340\,m\mu}$ until this was constant. The equilibrium concentration of DPNH was calculated from $E_{340\,m\mu}$ (Horecker & Kornberg, 1948); that of tartronic semialdehyde was taken to be equal to the concentration of DPNH thus determined. Since the amounts of glycerate and of $DPN^+$ utilized in the reaction were less than 1% of those added, the concentration of these reactants was assumed to be constant throughout. The pH was measured with a glass electrode.

The mean value obtained for $K$ was $2 \times 10^{-6}$ at pH 7·5 and $1·6 \times 10^{-5}$ at pH 8·5. This tenfold increase in $K$ accompanying a tenfold decrease in $H^+$-ion concentration was expected, as a decrease in $H^+$-ion concentration favours glycerate oxidation (reaction 2). In contrast, $K_H$ was independent of pH, and was determined as $5·8 \times 10^{-14}$ M at pH 7·5 and $5·1 \times 10^{-14}$ M at pH 8·5 (Table 4). Taking the latter figure as the most reliable, $\Delta G'$ at pH 7 was calculated to be +8·6 kcal./mole. Similarly, from the mean value of $K_H = 5·1 \times 10^{-14}$ M,

$$\Delta G^0 = +18 \text{ kcal./mole.}$$

Table 3. *Oxidation of glycerate coupled to reduction of pyruvate by crystalline enzymes*

The complete system contained, in 1 ml.: 50 $\mu$moles of potassium phosphate, pH 8·5; 0·5 $\mu$mole of $DPN^+$; 0·05 mg. of crystalline lactic dehydrogenase (Boehringer); 0·3 mg. of crystalline tartronic semialdehyde reductase; 25 $\mu$moles of sodium DL-glycerate; 1·0 $\mu$mole of potassium pyruvate. After incubation for 20 min. or 60 min. at 18°, samples (0·2 ml.) were removed and assayed for pyruvate content (Friedemann & Haugen, 1943).

| Reaction mixture | Pyruvate removed ($\mu$moles) In 20 min. | In 60 min. |
|---|---|---|
| Complete system | 0·37 | 0·60 |
| $DPN^+$ omitted | 0·01 | 0·05 |
| Lactic dehydrogenase omitted | 0 | 0 |
| Tartronic semialdehyde reductase omitted | 0·01 | 0·05 |
| Glycerate omitted | 0 | 0 |

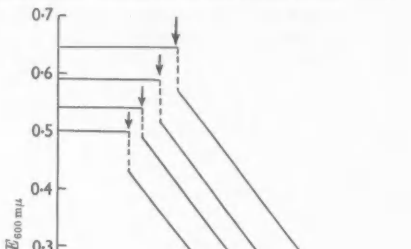


Fig. 5. Oxidation of D-glycerate, catalysed by crystalline tartronic semialdehyde reductase. The complete system contained, in 1 ml.: 50 $\mu$moles of potassium phosphate, pH 8·5; 10 $\mu$moles of KCN; 0·5 ml. of 0·01% 2:6-dichlorophenol-indophenol; 0·3 $\mu$mole of $DPN^+$; 0·4 $\mu$g. of crystalline tartronic semialdehyde reductase; 0·01 ml. of DPNH-dehydrogenase; 5 $\mu$moles of potassium D-glycerate. From each cuvette, one constituent had been omitted: this was added at the time indicated by the arrow. *A*, Glycerate omitted; *B*, $DPN^+$ omitted; *C*, tartronic semialdehyde reductase omitted; *D*, DPNH-dehydrogenase omitted. (Taken from a tracing made with a Cary recording spectrophotometer.)

Table 4. *Equilibrium constants for the oxidation of glycerate to tartronic semialdehyde*

The reaction mixture contained, in 1 ml.: 100 $\mu$moles of potassium phosphate, pH 7·5 or 8·5 as specified; 0·50 mg. of crystalline tartronic semialdehyde reductase; 1·50 or 3·50 $\mu$moles of $DPN^+$ as specified; various amounts of potassium D-glycerate. $E_{340\,m\mu}$ was measured until there was no further change. Concentrations of glycerate, $DPN^+$ and $H^+$ were assumed not to vary throughout the reaction and the concentration of tartronic semialdehyde formed was taken to be equal to that of the DPNH. $K$ and $K_H$ were calculated as described in the text.

| Equilibrium concn. (M) $10^3$[$DPN^+$] | $10^5$[DPNH] | $10^2$[glycerate] | $10^9$[$H^+$] | Calc. equilibrium constants $10^5K$ | $10^{14}$ M $\times K_H$ |
|---|---|---|---|---|---|
| 1·50 | 1·10 | 0·46 | 3·2 | 1·75 | 5·61 |
| 1·50 | 1·40 | 0·92 | 3·2 | 1·42 | 4·54 |
| 1·50 | 1·70 | 1·38 | 3·2 | 1·42 | 4·54 |
| 1·50 | 2·07 | 1·84 | 3·2 | 1·56 | 4·97 |
| 1·50 | 2·40 | 2·30 | 3·2 | 1·67 | 5·34 |
| 1·50 | 2·70 | 2·76 | 3·2 | 1·76 | 5·74 |
| | | | Average | 1·60 | 5·12 |
| 1·50 | 0·41 | 0·70 | 32 | 0·16 | 5·00 |
| 3·50 | 0·70 | 0·70 | 32 | 0·21 | 6·61 |
| | | | Average | 0·19 | 5·80 |

From the value of $\Delta G'$ obtained, the oxidation–reduction potential for reaction (2) is $-0{\cdot}228$ v. Since the oxidation–reduction potential of the system, $DPNH + H^+ \rightleftharpoons DPN^+ + 2H + 2e$, is $-0{\cdot}320$ v (Burton & Wilson, 1953), $E'_0$ of the reaction

$$\text{Glycerate}^- \rightleftharpoons \text{tartronic semialdehyde}^- + 2H + 2e$$

at pH 7 and 25° is $-0{\cdot}092$ v. This places the reaction among the more electropositive of known DPN-linked systems.

*Properties of the enzyme*

pH *optimum*. The crystalline enzyme catalysed the oxidation of DPNH by enzymically prepared tartronic semialdehyde over a wide range of $H^+$-ion concentration. The reaction proceeded at maximal velocity between pH 6·2 and pH 8·7, but even at pH 5, over 60 %, and at pH 10 over 30 %, of this rate was observed. With chemically prepared tartronic semialdehyde, the pH optimum appeared to lie further to the acid side, and assays with this substrate were done at pH 7·2.

*Effect of concentration of reactants on initial rate.* (*a*) Tartronic semialdehyde. The Michaelis constant ($K_m$) for enzymically prepared tartronic semialdehyde was determined from the plot of reciprocal rate of DPNH oxidation against reciprocal substrate concentration (Lineweaver & Burk, 1934) when various amounts of tartronic semialdehyde were reduced in the presence of an excess of crystalline enzyme and of DPNH at pH 8·5. The $K_m$ thus determined was $2 \times 10^{-4}$ M.

(*b*) Reduced pyridine nucleotides. At pH 8·5, the crystalline enzyme catalysed the reduction of tartronic semialdehyde approximately twice as rapidly with DPNH as with reduced TPN. The $K_m$ values at this pH, determined from the Lineweaver–Burk plot, obtained when a constant excess of enzyme and of tartronic semialdehyde reacted with various amounts of DPNH or TPNH, were $2 \times 10^{-5}$ M for DPNH and $5 \times 10^{-5}$ M for TPNH. At pH 6·5, TPNH was oxidized more rapidly and at a rate similar to that observed with DPNH.

(*c*) Glycerate. From the effect of various concentrations of D-glycerate on the initial rate of reduction of 2:6-dichlorophenol-indophenol, in the presence of an excess of crystalline tartronic semialdehyde reductase, DPNH-dehydrogenase, electron-acceptor dye and $DPN^+$ at pH 8·5, $K_m$ for D-glycerate was calculated to be $4 \times 10^{-4}$ M.

*Specificity for substrates.* The crystalline tartronic semialdehyde reductase did not catalyse the oxidation of DPNH in the presence of glyoxylate, oxaloacetate, pyruvate, glycolaldehyde, DL-glyceraldehyde, glyoxal, formaldehyde, reductone [CHO·CH(OH)·CHO] or mesoxalate ($CO_2^-$·CO·$CO_2^-$).

Some enzymic oxidation of DPNH was observed in the presence of dihydroxyfumarate. However, this reaction was not accompanied by any decrease in $E_{290\,m\mu}$, as would be expected from the utilization of dihydroxyfumarate: it is therefore likely that the dihydroxyfumarate solution used had partially decomposed to tartronic semialdehyde (Chow & Vennesland, 1958).

The crystalline enzyme catalysed the oxidation of DPNH or TPNH in the presence of hydroxypyruvate but had a lower affinity for this substrate than for tartronic semialdehyde. At saturating concentrations of substrates, the rate of oxidation observed with hydroxypyruvate was less than 17 % of that noted with tartronic semialdehyde, and, at concentrations sufficient to saturate the enzyme with respect to tartronic semialdehyde, the rate observed with hydroxypyruvate at pH 8·5 was less than 3 % (Fig. 6). This low rate of enzymic reduction of hydroxypyruvate to glycerate contrasts sharply with the similar reaction (5) catalysed by the D-glyceric dehydrogenase of plants (Stafford *et al.* 1954; Holzer & Holldorf, 1957). Whereas hydroxypyruvate was rapidly reduced in the presence of DPNH and this enzyme, tartronic semialdehyde did not react (Fig. 6):

$$HO{\cdot}CH_2{\cdot}CO{\cdot}CO_2H + DPNH + H^+ \rightarrow HO{\cdot}CH_2{\cdot}CH(OH){\cdot}CO_2H + DPN^+ \quad (5)$$

Malonic semialdehyde similarly reacted with the crystalline enzyme and reduced pyridine

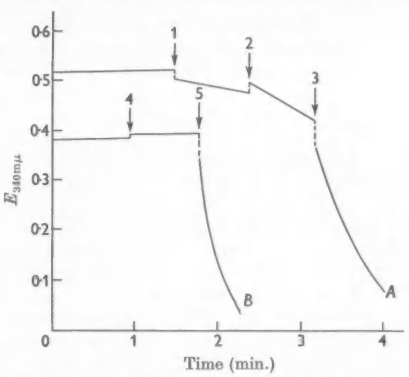


Fig. 6. Reaction of hydroxypyruvate and tartronic semialdehyde with tartronic semialdehyde reductase and D-glyceric dehydrogenase. The reaction mixtures contained, in 1·0 ml.: 50 $\mu$moles of potassium phosphate, pH 7·5; 0·1 $\mu$mole of DPNH; either tartronic semialdehyde reductase (0·10 mg.) or D-glyceric dehydrogenase. *A*, Tartronic semialdehyde reductase used, 1·2 $\mu$moles of hydroxypyruvate added at arrow 1, 90 $\mu$moles of hydroxypyruvate at arrow 2 and 1·2 $\mu$moles of tartronic semialdehyde at arrow 3; *B*, D-glyceric dehydrogenase used, 1·2 $\mu$moles of tartronic semialdehyde added at arrow 4 and 1·2 $\mu$moles of hydroxypyruvate at arrow 5. (Taken from a tracing made with a Cary recording spectrophotometer.)

nucleotides, but the observed oxidation proceeded 1·8 times as fast with DPNH as with TPNH. At saturating concentrations of substrates, the rate of DPNH oxidation at pH 8·5 was 13% of that observed with tartronic semialdehyde, and $K_m$ for malonic semialdehyde was calculated to be of the order of $2 \times 10^{-4}$ M.

The crystalline enzyme also catalysed the reduction of mesoxalic semialdehyde with concomitant oxidation of DPNH or TPNH. The product of this reaction was shown to be hydroxypyruvate:

$$CHO \cdot CO \cdot CO_2H + DPNH \text{ (or TPNH)} + H^+ \rightarrow HO \cdot CH_2 \cdot CO \cdot CO_2H + DPN^+ \text{ (or TPN}^+\text{)} \quad (6)$$

by the use of D-glyceric dehydrogenase (Fig. 7). Addition of this enzyme, which did not catalyse the reduction of tartronic semialdehyde (Fig. 6), to a reaction mixture in which DPNH oxidation in the presence of crystalline tartronic semialdehyde reductase and a limited quantity of mesoxalic semialdehyde had proceeded almost to completion, caused a further oxidation of DPNH. This effect was not observed when TPNH was used: it is known (Stafford *et al.* 1954) that the reduction of hydroxypyruvate to glycerate by D-glyceric dehydrogenase is specific for DPNH. Similarly, the rate of DPNH oxidation observed when mesoxalic semialdehyde was incubated with a mixture of crystalline tartronic semialdehyde reductase and D-glyceric dehydrogenase was greater than with tartronic semialdehyde reductase alone, as expected from the sum of reactions (5) and (6).

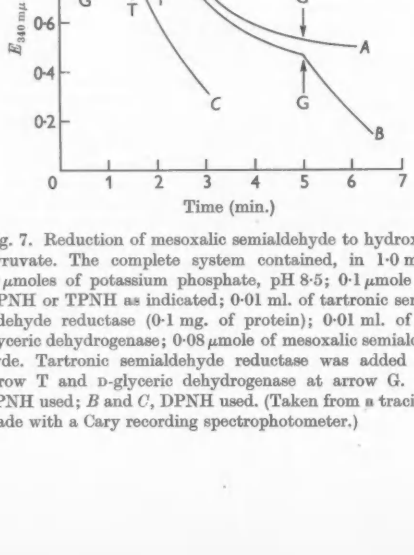


Fig. 7. Reduction of mesoxalic semialdehyde to hydroxypyruvate. The complete system contained, in 1·0 ml.: 50 $\mu$moles of potassium phosphate, pH 8·5; 0·1 $\mu$mole of DPNH or TPNH as indicated; 0·01 ml. of tartronic semialdehyde reductase (0·1 mg. of protein); 0·01 ml. of D-glyceric dehydrogenase; 0·08 $\mu$mole of mesoxalic semialdehyde. Tartronic semialdehyde reductase was added at arrow T and D-glyceric dehydrogenase at arrow G. *A*, TPNH used; *B* and *C*, DPNH used. (Taken from a tracing made with a Cary recording spectrophotometer.)

The crystalline tartronic semialdehyde reductase did not catalyse the reduction of 2:6-dichlorophenol-indophenol in the presence of DPNH-dehydrogenase and $DPN^+$ when incubated with L-malate, L-lactate, 3-phosphoglycerate, tartrate, glycollate or $\beta$-hydroxypropionate, but, apart from glycerate, reacted only with hydroxypyruvate (presumably by reversal of reaction 6).

*Inhibition of enzyme action.* The rate of reduction of tartronic semialdehyde by DPNH, catalysed by the crystalline enzyme, was not decreased in the presence of 1 mM-iodoacetate, of 10 mM-glycolaldehyde, DL-glyceraldehyde, sodium fluoride, 3-phosphoglycerate or of 4 mM-phosphoenolpyruvate. The rate of DPNH oxidation was slightly decreased in the presence of 10 mM-pyruvate, L-malate, formaldehyde, glyoxal, oxalate or tartronate, and was strongly inhibited by glyoxylate, glycollate or fluoroacetate (Table 5).

*Turnover number of the enzyme.* With DPNH as electron donor in the enzymic reduction of tartronic semialdehyde and at pH 8·5, the turnover number of the crystalline enzyme was 16 000 moles of DPNH oxidized/min./100 000 g. of protein. From

Table 5. *Inhibition of tartronic semialdehyde reductase*

Tartronic semialdehyde reductase was assayed as described in the Materials and Methods section (*a*), by measurement of the rate of oxidation of DPNH in the presence of tartronic semialdehyde and of the compounds listed below. The concentration of these added compounds in the reaction mixture was 10 mM unless indicated otherwise. Inhibition is expressed as:

$$\frac{\text{rate observed with tartronic semialdehyde and added compound}}{\text{rate observed with tartronic semialdehyde}} \times 100.$$

| Added compound | Rate (% of uninhibited rate) of oxidation |
|---|---|
| None | 100* |
| DL-Glyceraldehyde | 100 |
| Glycolaldehyde | 100 |
| Phosphoenolpyruvate (4 mM) | 100 |
| Sodium fluoride | 98 |
| 3-Phosphoglycerate | 95 |
| Iodoacetate (1 mM) | 95 |
| Glyoxal | 86 |
| L-Malate | 85 |
| Pyruvate | 85 |
| Formaldehyde | 79 |
| Oxalate | 78 |
| Tartronate | 74 |
| Glyoxylate | 52 |
| Fluoroacetate | 19 |
| Glycollate | 14 |

* This corresponded to a change in $E_{340\,m\mu}$ of 0·38 unit/min.

the estimated weight-average molecular weight ($M_w$) of 91 000 (see Appendix), this corresponds to a turnover number of 14 600 moles of DPNH oxidized/min./mole of enzyme.

*Stability.* The crystalline enzyme, stored as a suspension in ammonium sulphate, pH 7·5, at 2°, lost less than 10 % of its activity over a month.

## DISCUSSION

The metabolic routes whereby *Pseudomonas* spp. grow on glycollate or on other precursors of glyoxylate have been shown to involve the formation of glycerate from glyoxylate (Kornberg & Gotto, 1959, 1961). This synthesis of a $C_3$ acid from the $C_2$ substrate is a process involving two enzymic steps. In the first of these (reaction 1), 2 molecular units of glyoxylate undergo a decarboxylative condensation to yield a molecular unit each of carbon dioxide and of a $C_3$ compound which was postulated to be hydroxypyruvate (Krakow & Barkulis, 1956) or its isomer, tartronic semialdehyde. In the second enzymic step, the $C_3$ compound is reduced to glycerate.

Although authentic tartronic semialdehyde was not then available for comparison, the properties of the $C_3$ compound enzymically formed in reaction (1), which were listed by Kornberg & Gotto (1959, 1961), showed that it was not hydroxypyruvate and indicated that it was tartronic semialdehyde, which was supported by the findings of Krakow *et al.* (1959, 1961). This view is strengthened by the isolation of the crystalline enzyme catalysing reaction (2), and by the study of its properties, as reported in this paper.

In particular, both crude extracts of glycollate-grown *Pseudomonas* and the crystalline enzyme have been found to react with equal facility with the $C_3$ compound enzymically formed and with authentic tartronic semialdehyde, prepared by Professor D. B. Sprinson. Neither crude bacterial extracts nor the crystalline enzyme were found to catalyse the rapid reduction of hydroxypyruvate which would be expected were hydroxypyruvate an intermediate in growth on glycollate.

The crystalline enzyme, which catalyses the reduction of tartronic semialdehyde to glycerate and its reversal, may be distinguished from the other known glycerate-forming enzyme, the D-glyceric dehydrogenase of plants (Stafford *et al.* 1954; Holzer & Holldorf, 1957), on the basis of specificity for substrate and for coenzyme. Whereas the bacterial enzyme reacts rapidly with tartronic semialdehyde but only slowly with hydroxypyruvate, the plant enzyme reacts rapidly with hydroxypyruvate and does not react with tartronic semialdehyde. Moreover, unlike the bacterial enzyme, the plant enzyme also catalyses the reduction of glyoxylate. The crystalline bacterial enzyme reacts with TPNH at half the rate at which it reacts with DPNH, and the $K_m$ values for those two coenzymes are of comparable magnitude ($5 \times 10^{-5}$ M and $2 \times 10^{-5}$ M respectively): the plant enzyme reacts only with DPNH. On the basis of these findings, it was proposed (Gotto & Kornberg, 1961*a*) to name the bacterial glycerate-forming enzyme 'tartronic semialdehyde reductase'.

The crystalline tartronic semialdehyde reductase differed in its substrate specificity also from other enzymes catalysing the reduction of :C:O linkages. For example, alcohol dehydrogenases are known to exhibit low substrate specificity (Dixon & Webb, 1958), commercial crystalline lactic dehydrogenase of muscle was found readily to reduce hydroxypyruvate and glyoxylate as well as pyruvate, and glyoxylate reductase of tobacco leaves (Zelitch, 1955) reduced hydroxypyruvate as well as glyoxylate. None of these enzymes reacts with tartronic semialdehyde. Furthermore, the crystalline tartronic semialdehyde reductase does not catalyse the reduction of pyruvate or glyoxylate, and indeed is inhibited by this latter substrate, but was found to react rapidly only with $C_3$ compounds of general structure $CHO \cdot R \cdot CO_2H$. Thus besides tartronic semialdehyde (R = $CH \cdot OH$), only malonic semialdehyde (R = $CH_2$) and mesoxalic semialdehyde (R = CO) were found to be enzymically reduced. It is possible that the slow reduction of hydroxypyruvate at pH 8·5 is a result of its slow isomerization to tartronic semialdehyde (cf. Chow & Vennesland, 1958).

## SUMMARY

1. Tartronic semialdehyde reductase was purified 200-fold and crystallized from glycollate-grown *Pseudomonas ovalis* Chester.

2. The enzyme catalyzed the reduction of tartronic semialdehyde to glyceric acid with reduced di- or tri-phosphopyridine nucleotide as electron donor. $K_m$ for tartronic semialdehyde at pH 8·5 was measured to be $2 \times 10^{-4}$ M. $K_m$ for reduced diphosphopyridine nucleotide under these conditions was $2 \times 10^{-5}$ M and for reduced triphosphopyridine nucleotide $5 \times 10^{-5}$ M.

3. The crystalline enzyme oxidized 160 $\mu$moles of reduced diphosphopyridine nucleotide/min./mg. of protein at pH 8·5, 23°, in the presence of tartronic semialdehyde. On the basis of the measured weight-average molecular weight $M_w$ of 91 000, this gave a turnover number of 14 600 moles of reduced diphosphopyridine nucleotide oxidized/min./mole of enzyme.

4. The enzyme was shown also to catalyse the oxidation of glycerate with concomitant reduction of oxidized diphosphopyridine nucleotide. The

equilibrium constant $K$ at 23° was $2 \times 10^{-6}$ at pH 7·5 and $1{\cdot}6 \times 10^{-5}$ at pH 8·5; $K_H$ was calculated to be $5{\cdot}1 \times 10^{-14}$ M. From these data, $\Delta G'$ at pH 7, 25°, was determined as 8·6 kcal./mole and $\Delta G°$ as 18 kcal./mole. Similarly, the oxidation–reduction potential for the reaction

$$\text{Glycerate}^- \rightleftharpoons \text{tartronic semialdehyde}^- + 2H + 2e$$

was found to be $E'_0 = -0{\cdot}092$ v at 25° and pH 7.

5. The enzymic oxidation of glycerate to tartronic semialdehyde was also demonstrated by coupling it to the reduction of pyruvate to lactate, in the presence of oxidized diphosphopyridine nucleotide and crystalline lactic dehydrogenase, and to the reduction of 2:6-dichlorophenol-indophenol in the presence of oxidized diphosphopyridine nucleotide and reduced diphosphopyridine nucleotide dehydrogenase. The latter system served as a convenient assay of the enzyme.

6. The enzyme catalysed the reduction only of $C_3$ compounds of structure $CHO \cdot R \cdot CO_2H$, where R = $CH \cdot OH$, $CH_2$ or CO. Tartronic semialdehyde (R = $CH \cdot OH$) reacted more than five times as fast as any other substrate.

7. The enzyme catalysed the oxidation only of glyceric acid and of hydroxypyruvate.

8. The significance of these findings to the pathways of biosynthesis from glyoxylate is discussed.

We thank Professor Sir Hans Krebs, F.R.S., for his interest and encouragement, Professor D. B. Sprinson for preparing authentic tartronic semialdehyde, Dr I. Zelitch for collaborating in parts of this work, Miss A. Taylor for technical assistance and Mr R. Elsworth for providing mass cultures of glycollate-grown *Pseudomonas ovalis* Chester. We are also greatly indebted to Dr R. Barer for the photograph (Fig. 2). This work was done while A.M.G. was a Pre-doctoral Fellow of the National Science Foundation, Washington, D.C. (U.S.A.), and was supported by the Rockefeller Foundation, by the U.S. Public Health Service and by the Office of Scientific Research of the Air Research and Development Command of the United States Air Force, through its European Office, under Contract no. AF 61 (052)–180.

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APPENDIX

# Sedimentation Behaviour of the Enzyme

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(*Received* 24 *April* 1961)

The sedimentation behaviour of the crystalline tartronic semialdehyde reductase was examined in a Spinco model E analytical ultracentrifuge. The enzyme was dissolved in, and dialysed against, 0·1 M-sodium potassium phosphate buffer, pH 7·60, for 21 hr. at 3°. The protein concentration of the dialysed solution was measured refractometrically and, assuming a specific refractive increment of $1{\cdot}86 \times 10^{-3}$, was 0·765 g./100 ml. of solution.

The enzyme sedimented in the ultracentrifuge as a single symmetrical peak (Fig. 3, p. 277) with $S_{20,w}$ $6{\cdot}14 \times 10^{-13}$ sec. at a protein concentration of 0·765 g./100 ml. of solution. At least 90 % of the protein in the ultracentrifuge cell could be accounted for by the area under the schlieren peak at all times during the sedimentation. The areas were corrected for radial dilution. The variation of sedimentation coefficient ($S_{20,w}$) with protein concentration appeared anomalous, showing a maximum value of $6{\cdot}68 \times 10^{-13}$ sec. at a protein concentration of about 0·28 g./100 ml. This may be an indication of a possible association phenomenon, as found for chymotrypsin by Schwert (1949), although insufficient material was available for this point to be examined in detail.

The weight-average molecular weight ($M_w$) was measured by the Archibald (1947) technique under the following conditions: rotor speed 7447 rev./min., temperature 1·8°, protein concentration 0·765 g./100 ml. Photographs were taken at 10 min. intervals at a phase-plate angle on the instrument of 70°. The mean molecular weight ($M_w$) was found to be $90\,800 \pm 1300$ (S.E.M. four photographs). No significant difference was observed between measurements made at the top and at the bottom of the cell.

The preparation was examined for homogeneity by computing the apparent diffusion coefficient ($D_{app.}$) from the sedimentation patterns and examining the variation of this quantity with time. $D_{app.}$ was computed from the equation

$$D_{app.} = \frac{1}{4\pi t}\left(\frac{A}{H_{max.}}\right)^2 . (1 - \omega^2 S . t)$$

where $A$ is the area under the schlieren peak, $H_{max.}$ the maximum ordinate of the peak, $S$ the sedimentation coefficient ($S_{20,w}$) and $t$ the time (sec.). Computed valves of $D_{app.}$ did not show any variation with time and gave a value of $6{\cdot}3 \times 10^{-7}$ cm.$^2$ sec.$^{-1}$ (uncorr.) at a protein concentration of 0·096 g./100 ml. of solution. Measurement of $D_{app.}$ at a higher protein concentration (0·765 g./100 ml.) gave an anomalous result, decreasing rapidly with time and later levelling off, indicating that it is necessary to apply corrections for the effect of $c$ upon $S$ at this concentration (Baldwin, 1957; Schachman, 1959). It is concluded that, at low protein concentration, no inhomogeneity is revealed by this test.

Tests for the presence of metal ions were done after digestion of 2 mg. of the crystalline enzyme in 5 ml. of concentrated nitric acid for 20 min. at 100°. The digest, examined with the flame-photometer attachment of the Beckman DU spectrophotometer, was found not to contain significant amounts of Zn, Ca, Mn, Fe, Cu and Mg.

## SUMMARY

1. A preparation of crystalline tartronic semialdehyde reductase was examined in the ultracentrifuge and appeared to be homogeneous at low protein concentration.

2. A value of $S_{20,w}$ of $6{\cdot}14 \times 10^{-13}$ sec. at 0·765 % (w/v) protein concentration was obtained; the molecular weight was found to be 90 800.

3. Zinc, calcium, manganese, iron, copper and magnesium were not detected in the preparation by flame photometry.

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*Biochem. J.* (1961) **81**, 286

# Effect of Protein and Fatty Acids on the Oxidation of Succinate and α-Oxoglutarate by Pea-Root Mitochondria

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(*Received* 4 *April* 1961)

The oxidation of succinate and α-oxoglutarate by pea-root mitochondria was increased by cytochrome *c* or bovine-plasma albumin (Stickland, 1960). If the mitochondria were first shaken at 25° much higher rates of oxygen uptake were obtained. Similar effects of protein on oxidative phosphorylation have been shown in mitochondria from insects (Wojtczak & Wojtczak, 1960; Lewis & Fowler, 1960) and from liver (Hülsmann, Elliott & Slater, 1960). The activity of U factor, an uncoupling agent found by Lehninger & Remmert (1959) in rat-liver mitochondria, was abolished by plasma albumin. Since long-chain fatty acids could be extracted from protein that had been incubated with these mitochondria, it was suggested that the protein reversed an inhibition by fatty acids, and some fatty acids were shown to be inhibitory.

A similar effect appears to be operative in pea roots. In the present paper a further study of the effect of these proteins and of several fatty acids is reported.

## EXPERIMENTAL

Peas were grown under sterile conditions and mitochondria extracted as reported by Stickland (1960). The terminal 2 cm. of pea roots was homogenized in 1 vol. of 0·6 M-sucrose in 0·05 M-phosphate buffer, pH 6·8, at 0°. Occasionally other lengths of root were used. The homogenate was centrifuged at 1000*g* for 10 min. and the supernatant centrifuged again at 15 000*g* for 15 min. The precipitate, which was composed mainly of mitochondria, was washed by resuspension in 0·5 vol. of the homogenizing medium, centrifuged again and finally resuspended in 0·5 vol. of medium.

In some experiments, after the second centrifuging the mitochondria were washed twice with 0·35 vol. of the homogenizing medium containing 3% (w/v) bovine-plasma albumin, and then washed once with 0·5 vol. of homogenizing medium and resuspended as above. All volumes are relative to the initial homogenizing volume.

Oxygen uptake was measured at room temperature (21–23°) by a polarographic method with a stationary platinum electrode sheathed with a thin polythene sheet (see Stickland, 1960). The reaction medium contained 0·05 M-phosphate buffer, pH 6·8, 0·1 ml. of mitochondrial suspension and other additions as stated, in a total volume of 0·5 ml.

Nitrogen estimations were done by the method of Sunderland, Heyes & Brown (1957) on the 'acid-insoluble matter' obtained by the procedure of Werkheiser & Bartley (1957). Oxygen uptake is expressed as $q_{O_2}$ (μl. of oxygen/mg. of N/hr.).

*Preparation of ether extracts.* Extracts of mitochondria, of bovine-plasma albumin that had been incubated with mitochondria and then precipitated with 2% (v/v) perchloric acid, and of the supernatant from this precipitation were prepared by shaking the material with four lots of 2 ml. of diethyl ether at 30°. The ether extracts were pooled and evaporated to dryness under a stream of nitrogen, and the residue was suspended or dissolved in water. Usually the weight of extracted material added to the reaction medium was equivalent to the weight originally present in 0·3 ml. of mitochondrial suspension.

*Special chemicals.* The following were used: bovine-plasma albumin and bovine γ-globulin (Armour Laboratories); diphosphopyridine nucleotide and linolenic acid (L. Light and Co. Ltd., Colnbrook, Bucks.); adenosine diphosphate and cytochrome *c* (Sigma Chemical Co., St Louis, Mo., U.S.A.); linoleic acid (British Drug Houses Ltd.).

## RESULTS

*Effect of cytochrome c on succinate oxidation.* Addition of cytochrome *c* up to a concentration of 0·1 mM had no effect on the endogenous oxygen uptake of either unshaken mitochondria or mitochondria that were first shaken in a test tube in a water bath at 25° for 30 min. (Fig. 1*A*). There was very little oxidation of succinate in the absence of added cytochrome *c*, but as the cytochrome *c* concentration was increased the rate of oxidation rose, the increase with shaken mitochondria being much greater than that with unshaken mitochondria.

*Effect of bovine-plasma albumin.* Similar results were obtained with bovine-plasma albumin (Fig. 1*B*). The difference in $q_{O_2}$ for succinate obtained with unshaken and shaken mitochondria was more marked than with cytochrome *c*. Bovine-plasma albumin had a similar effect on the oxidation of α-oxoglutarate (Table 1). There was only a very slow rate of oxidation of α-oxoglutarate by unshaken mitochondria but this was increased by bovine-plasma albumin. With shaken mitochondria there was appreciable oxidation of α-oxoglutarate and this was increased very considerably by bovine-plasma albumin, but in all cases the rate of oxygen uptake was less than with succinate as substrate.

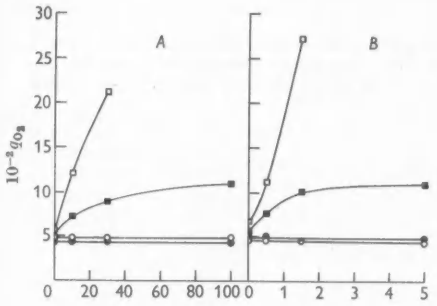


Fig. 1. Effect of cytochrome *c* and bovine-plasma albumin on succinate oxidation by unshaken and shaken mitochondria. The reaction medium contained 0·05 M-phosphate buffer, pH 6·8, 0·1 ml. of mitochondrial suspension and succinate (10 mM) and cytochrome *c* (*A*) or bovine-plasma albumin (*B*) as indicated, in a total volume of 0·5 ml. ●, Endogenous, unshaken mitochondria; ○, endogenous, shaken mitochondria; ■ plus succinate, unshaken mitochondria; □, plus succinate, shaken mitochondria.

Table 1. *Effect of bovine-plasma albumin on the oxidation of α-oxoglutarate by unshaken and shaken mitochondria*

Reaction medium contained 0·05 M-phosphate buffer, pH 6·8, 0·1 ml. of mitochondrial suspension and additions as below in a total volume of 0·5 ml.

| Additions | $q_{O_2}$ Unshaken mitochondria | $q_{O_2}$ Shaken mitochondria |
|---|---|---|
| None | 490 | 510 |
| α-Oxoglutarate (10 mM) | 510 | 760 |
| Bovine-plasma albumin (5 mg./ml.) | 520 | 490 |
| α-Oxoglutarate (10 mM) + bovine-plasma albumin (5 mg./ml.) | 710 | 1070 |

*Ether extract of mitochondria.* An ether extract of pea-root mitochondria inhibited succinate oxidation. For this experiment the mitochondria were washed twice with homogenizing medium containing 3% (w/v) bovine-plasma albumin, and then once with homogenizing medium alone to remove excess of albumin. Mitochondria prepared in this way oxidized succinate at a rapid rate without the addition of bovine-plasma albumin, and the addition of 1·5 mg. of albumin/ml. did not affect the rate (Table 2). Ether extract corresponding to 0·3 ml. of mitochondrial suspension had no effect on the endogenous rate but resulted in 90% inhibition of succinate oxidation. This inhibition was completely reversed by the addition of bovine-plasma albumin.

Table 2. *Effect of ether extract of mitochondria on the oxidation of succinate by unshaken mitochondria*

Mitochondria were prepared in a sucrose–phosphate medium, washed twice with a medium containing 3% (w/v) bovine-plasma albumin and washed once with and finally resuspended in sucrose–phosphate. Reaction medium contained 0·05 M-phosphate buffer, pH 6·8, 0·1 ml. of mitochondrial suspension (unshaken) and additions as below in a total volume of 0·5 ml. The amount of mitochondrial extract originating from 0·3 ml. of mitochondrial suspension was used in each case.

| Additions | $q_{O_2}$ No extract | $q_{O_2}$ Mitochondrial extract |
|---|---|---|
| None | 530 | 550 |
| Succinate (10 mM) | 1160 | 610 |
| Bovine-plasma albumin (1·5 mg./ml.) | 530 | 560 |
| Succinate (10 mM) + bovine-plasma albumin (1·5 mg./ml.) | 1170 | 1160 |

*Ether extract of bovine-plasma albumin.* A mitochondrial preparation was suspended in an equal volume of 3% (w/v) bovine-plasma albumin at room temperature for 5 min. The mitochondria were removed by centrifuging and the protein was precipitated from the supernatant with 2% (v/v) perchloric acid. Ether extracts were prepared (see Experimental section) of the sedimented mitochondria, of the precipitated albumin and of the final supernatant.

The effect of the extract of the albumin on the oxidation of succinate by mitochondria previously washed with bovine-plasma albumin is shown in Expt. 1 of Table 3. Approximately 50% inhibition of succinate oxidation was obtained and this inhibition was completely reversed by the addition of bovine-plasma albumin. The extract had no effect on the endogenous oxygen uptake.

Succinate oxidation was not inhibited by the ether extracts of the mitochondria that had been incubated with bovine-plasma albumin (see above), by the ether extract of the supernatant from the precipitated albumin or by a suspension in water of the precipitated albumin that had been extracted with ether.

If shaken albumin-washed mitochondria were used, the inhibition of succinate oxidation by the ether extract of bovine-plasma albumin was only about 20% (Table 3, Expt. 2). With α-oxoglutarate as substrate the inhibition by the albumin extract was about 75% with both unshaken and shaken mitochondria (Table 3, Expts. 3 and 4). All these

Table 3. *Effect of ether extract of bovine-plasma albumin on the oxidation of succinate and α-oxoglutarate*

Mitochondria were prepared and washed as noted in Table 2, and the reaction medium was as in Table 1. Concn. of succinate and α-oxoglutarate was 10 mM and that of bovine-plasma albumin was 1·5 mg./ml. The amount of bovine-plasma albumin extract originating from 0·3 ml. of mitochondrial suspension was used in each case.

| Expt. no. | Mitochondria | Additions | $q_{O_2}$ No extract | $q_{O_2}$ Albumin extract |
|---|---|---|---|---|
| 1 | Unshaken | None | 540 | 520 |
| | | Succinate | 1430 | 980 |
| | | Bovine-plasma albumin | 540 | 520 |
| | | Succinate + bovine-plasma albumin | 1490 | 1460 |
| 2 | Shaken | None | 490 | 470 |
| | | Succinate | 1270 | 1100 |
| | | Bovine-plasma albumin | 470 | 460 |
| | | Succinate + bovine-plasma albumin | 1280 | 1240 |
| 3 | Unshaken | None | 500 | 510 |
| | | α-Oxoglutarate | 630 | 540 |
| | | Bovine-plasma albumin | 500 | 500 |
| | | α-Oxoglutarate + bovine-plasma albumin | 650 | 630 |
| 4 | Shaken | None | 490 | 470 |
| | | α-Oxoglutarate | 670 | 520 |
| | | Bovine-plasma albumin | 470 | 480 |
| | | α-Oxoglutarate + bovine-plasma albumin | 690 | 680 |

Table 4. *Effect of bovine γ-globulin on the oxidation of succinate by unshaken and shaken mitochondria*

Reaction medium as given in Table 1. Concn. of succinate was 10 mM and of bovine γ-globulin 1·5 mg./ml.

| Additions | $q_{O_2}$ Unshaken mitochondria | $q_{O_2}$ Shaken mitochondria |
|---|---|---|
| None | 500 | 470 |
| Succinate | 600 | 680 |
| γ-Globulin | 510 | 490 |
| Succinate + γ-globulin | 690 | 780 |

inhibitions were completely reversed by bovine-plasma albumin.

An ether extract of 3% (w/v) bovine-plasma albumin alone inhibited succinate oxidation about 5%.

*Effect of γ-globulin.* Bovine γ-globulin, at a concentration of 1·5 mg./ml., also increased the rate of succinate oxidation by both unshaken and shaken mitochondria (Table 4), but the effect was very much less than that given by the same concentration of albumin. A higher concentration (5 mg./ml.) of γ-globulin caused inhibition of succinate oxidation.

*Effect of fatty acids.* The effect of five fatty acids on the oxidation of succinate by unshaken mitochondria is given in Table 5.

Stearate, octanoate, linoleate and linolenate all increased the oxygen uptake in the presence or absence of 10 mM-succinate. There was inhibition of succinate oxidation ($h-g$ in Table 5) by 1 and 10 μM-linoleate, but owing to the rise in the endogenous rate there was no inhibition of the total oxygen uptake in the presence of succinate. At a concentration of 10 μM the other three fatty acids had little effect on succinate oxidation.

However, oleate depressed the total oxygen uptake in the presence of succinate, resulting in a marked inhibition of succinate oxidation ($f-e$ in Table 5). Oleate (10 μM) also increased the endogenous oxygen uptake.

Addition of bovine-plasma albumin tended to reverse the effects of stearate and oleate: wherever the oxygen uptake was increased by the fatty acids, albumin reduced it. However, in those cases where oleate decreased the oxygen uptake, addition of albumin raised it.

Large increases in the oxygen uptakes with or without succinate were caused by higher concentrations (up to 10 mM) of all five fatty acids. These were perhaps due to oxidation of the fatty acids themselves or to the uncoupling of oxidative phosphorylation, which fatty acids are now thought to do (see Lehninger & Remmert, 1959; Wojtczak & Wojtczak, 1960).

*Different concentrations of ether extract.* The effect of three concentrations of ether extract is also shown in Table 5. The two lower concentrations had no effect in the endogenous rate but inhibited succinate oxidation. The higher concentration, obtained from a different mitochondrial preparation, increased the oxygen uptake in both the absence and presence of succinate and caused some stimulation of succinate oxidation. Bovine-plasma albumin reversed the inhibition of succinate oxidation.

Table 5. *Effect of fatty acids on succinate oxidation*

Mitochondria were prepared and incubated as given in Table 2. Concn. of succinate was 10 mM and that of bovine-plasma albumin 1·5 mg./ml. Other additions as below. The oxygen uptake is expressed relative to that obtained in the presence of succinate alone, which was made equal to 100. The oxygen uptake due to the oxidation of succinate, after subtraction of the endogenous rate, is given in the third column in each case.

| | Relative oxygen uptake with addition of | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Stearate | | | | | Octanoate | | | Oleate | | | | |
| Concn. of fatty acid (μM) | Alone *a* | With succinate *b* | *b* − *a* | With albumin | With succinate + albumin | Alone *c* | With succinate *d* | *d* − *c* | Alone *e* | With succinate *f* | *f* − *e* | With albumin | With succinate + albumin |
| 0 | 36 | 100 | 64 | 36 | 100 | 40 | 100 | 60 | 27 | 100 | 73 | 31 | 101 |
| 0·1 | — | 103 | — | — | — | 56 | 115 | 59 | — | 100 | — | — | — |
| 1 | 48 | 113 | 65 | 43 | 110 | 60 | 119 | 59 | 28 | 66 | 38 | 28 | 91 |
| 10 | 61 | 124 | 63 | 48 | 107 | 62 | 117 | 55 | 43 | 94 | 51 | 40 | 99 |

| | Relative oxygen uptake with addition of | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Linoleate | | | Linolenate | | | Ether extract | | | | | |
| Concn. of fatty acid (μM) | Alone *g* | With succinate *h* | *h* − *g* | Alone *i* | With succinate *j* | *j* − *i* | Alone *k* | With succinate *l* | *l* − *k* | With albumin | With succinate + albumin | Concn. of ether extract (μg. dry wt./ml. of reaction medium) |
| 0 | 53 | 100 | 47 | 41 | 100 | 59 | 36 | 100 | 64 | 37 | 96 | 0 |
| 0·1 | 53 | 100 | 47 | 41 | 100 | 59 | 39 | 73 | 34 | 40 | 100 | 19 |
| 1 | 66 | 103 | 37 | 51 | 109 | 58 | 36 | 82 | 46 | 36 | 96 | 190 |
| 10 | 80 | 109 | 29 | 77 | 140 | 63 | 55 | 128 | 73 | — | — | 292* |

* From a different mitochondrial preparation from the lower concentrations.

Table 6. *Distribution of mitochondrial nitrogen and of ether-extractable material along the root*

Mitochondria were prepared from four consecutive 5 mm. sections of 475 pea roots. After removal of a sample for nitrogen determinations, each mitochondrial suspension was added to an equal volume of 3% (w/v) bovine-plasma albumin and an ether extract of the latter prepared as described in the Experimental section. The effect of each ether extract on the oxidation of succinate by mitochondria (prepared from root tips, length 2 cm.) washed with albumin was determined and is given in the Table under $q_{O_2}$. The amount of extract originating from 0·3 ml. of mitochondrial suspension was used in each case. The figures for % dry weight of root sections were obtained from a different lot of roots.

| Section no. | Distance from root tip (mm.) | Fresh wt. of roots (g.) | Mitochondrial N ($\mu$g./475 sections) | Mitochondrial N ($\mu$g./g. fresh wt. of roots) | Dry wt. of ether extract ($\mu$g.) | Dry wt. of extract ($\mu$g./$\mu$g. of mitochondrial N) | $q_{O_2}$ Endogenous | $q_{O_2}$ With succinate | Dry wt. of root sections (%) |
|---|---|---|---|---|---|---|---|---|---|
| — | — | — | — | — | — | — | 450* | 1120* | — |
| 1 | 0–5 | 2·05 | 375 | 183 | <50 | <0·2 | — | 1130 | 16·1 |
| 2 | 5–10 | 2·74 | 220 | 80 | 157 | 0·7 | — | 1100 | 7·6 |
| 3 | 10–15 | 3·19 | 248 | 78 | 379 | 1·5 | 470 | 1000 | 6·2 |
| 4 | 15–20 | 4·02 | 328 | 82 | 1375 | 4·2 | 550 | 760 | 6·5 |

* No ether extract added.

Table 7. *Effect of bovine-plasma albumin on the oxidation of succinate by mitochondria isolated from the tip (1 cm.) of pea roots*

Mitochondria were prepared from the tip (1 cm. only) of pea roots, and were not washed with albumin. The reaction medium was as in Table 1.

| Additions | $q_{O_2}$ Unshaken mitochondria | $q_{O_2}$ Shaken mitochondria |
|---|---|---|
| None | 550 | 510 |
| Succinate (10 mM) | 1160 | 1820 |
| Bovine-plasma albumin (1·5 mg./ml.) | 540 | 580 |
| Succinate (10 mM) + bovine-plasma albumin (1·5 mg./ml.) | 1170 | 1810 |

*Mitochondrial nitrogen and ether-extractable material in pea-root sections.* Mitochondria were prepared from consecutive 5 mm. sections of pea roots. An equal volume of 3% (w/v) bovine-plasma albumin was added to each mitochondrial suspension, and an ether extract of the albumin made as described in the Experimental section. Mitochondrial protein nitrogen, the dry weight of the ether extract and the inhibitory effect of the ether extract on succinate oxidation by mitochondria prepared from 2 cm. lengths of root and washed with albumin are reported in Table 6. There was a twofold increase in the fresh weight of the roots from sections 1 to 4. Both the mitochondrial nitrogen per gram fresh weight of roots and the percentage dry weight of the root sections decreased to under half their value from sections 1 to 2 and then remained approximately constant. The total dry weight of the ether extract increased very greatly from sections 1 to 4 and this was accompanied by an increasing inhibition of succinate oxidation. The ether extract from section 4 also caused an increased oxygen uptake in the absence of succinate. The ratio of dry weight of extract to mitochondrial nitrogen increased at least 20 times from sections 1 to 4.

Since there was little ether-extractable material in the first two 5 mm. sections of the roots, it might have been expected that mitochondria prepared from 1 cm. sections should show no response to the addition of bovine-plasma albumin. This was in fact so, as indicated in Table 7. Addition of albumin had no effect on the oxygen uptake of either unshaken or shaken mitochondria.

*Oxygen uptake on a dry-weight basis.* Since many measurements of oxygen uptake are on a dry-weight basis, the dry weights of 2 cm.-long pea roots were determined to enable the results in this paper to be compared with others. With these dry-weight values, the endogenous oxygen uptake was about 0·5 $\mu$l. of oxygen/mg. dry wt./hr., and with succinate values up to 3·5 $\mu$l. of oxygen/mg. dry wt./hr. were obtained, depending upon the conditions.

## DISCUSSION

The work reported in this paper suggests that a substance present in pea roots inhibits the oxidation of succinate and α-oxoglutarate by mitochondria isolated from these roots. This inhibition could be reversed by the addition of protein (cytochrome *c* or bovine-plasma albumin) and the inhibitor could be extracted from the protein that had been incubated with the mitochondria. Stimulation of the mitochondrial oxidations by protein thus appeared to be due to removal of inhibitor by the protein. The inhibitor, possibly in a combined form, may occur in other parts of the cell and only become associated with the mitochondria on disintegration of the root cells. That the amount of

inhibitor associated with the mitochondria varied so greatly from one region of the root to another (see Table 6) suggests that this might be so. Since most inhibitor was found in those areas of the root that have highly vacuolated cells, the inhibitor might be released on homogenization of the tissue either because it is in the vacuole or because the acids known to be in the vacuole bring about its formation by hydrolysis.

The work of Hülsmann *et al.* (1960) suggests that in animal mitochondria the uncoupler of oxidative phosphorylation is one (or more) unsaturated fatty acid. Of the fatty acids whose effects on pea-root mitochondria have been studied, only oleic acid resembles the inhibitor in its action of inhibiting the total oxygen uptake in the presence of succinate. A higher concentration of linoleate caused inhibition of succinate oxidation, but the total oxygen uptake was greater than that without linoleate. It is thus possible that the inhibitor is oleic or a closely related acid. According to Meara (1957), in most roots 80–90 % of the fatty acids are unsaturated and consist mainly of oleic acid. The increased oxygen uptakes at high concentration of fatty acid are perhaps due partly to oxidation of the fatty acids themselves and partly to the uncoupling of oxidative phosphorylation, which fatty acids are now thought to do (see Lehninger & Remmert, 1959; Wojtczak & Wojtczak, 1960).

Particles isolated from cotton hypocotyls have been found to contain a substance that inhibits succinate oxidation (Throneberry, 1960), and this effect is partly reversed by plasma albumin. This inhibitory substance might be similar to the pea-root inhibitor.

The figures for distribution of mitochondrial nitrogen along the pea root are similar to those obtained for maize roots by Lund, Vatter & Hanson (1958). By using the values of Brown & Broadbent (1950) for the number of cells in pea roots, the amount of mitochondrial nitrogen per cell in sections 1 and 2 of the root is 3·6 and 8·2 $\mu\mu$g., which represent 2 and 3 % of the protein nitrogen per cell respectively. However, these are only minimal values since it is almost certain that a quantitative extraction of the mitochondria was not obtained. The ratio of mitochondrial nitrogen (per gram fresh weight of roots) to percentage dry weight of the roots is approximately constant in all the sections.

## SUMMARY

1. The oxidation of succinate and α-oxoglutarate by mitochondria from the terminal 2 cm. of pea roots was increased by cytochrome *c* or bovine-plasma albumin. If the mitochondria were washed with albumin, the rates of oxidation were high and addition of albumin caused no further increase.

2. A diethyl ether extract of the mitochondria, or of bovine-plasma albumin that had been incubated with the mitochondria, inhibited the oxidation of both substrates by albumin-washed mitochondria, and this inhibition could be reversed by addition of albumin.

3. Shaking the mitochondria for ½ hr. at 25° increased the rates of oxidation, but the effects of cytochrome *c*, bovine-plasma albumin and the ether extracts were still apparent.

4. Stearic, octanoic, oleic, linoleic and linolenic acids caused an increase of the oxygen uptake in the absence of succinate and in some cases a small inhibition or stimulation of succinate oxidation at high concentrations of fatty acid. The effect of ether extract was only similar to that produced by oleic acid, which brought about considerable inhibition of succinate oxidation at low concentration ($\mu$M).

5. Mitochondrial nitrogen (*a*) decreased and the amount of ether-extractable material (*b*) increased with increasing distance from the root tip. The ratio *b*/*a* increased 20-fold along the first 2 cm. of root. Oxidation of succinate by mitochondria isolated from the terminal 1 cm. of root was not affected by the addition of albumin.

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# The Effect of Hypophysectomy and Growth-Hormone Treatment of the Rat on the Incorporation of Amino Acids into Isolated Liver Ribosomes

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Hypophysectomy of the rat results in changes in liver microsomes so that they are less able to incorporate amino acids into protein *in vitro* than are those from normal rat liver (Korner, 1959*a*). Treatment of the rats with ox growth hormone can reverse this change to some extent. Experiments on amino acid incorporation into liver-microsome protein in whole animals (Korner, 1959*b*) confirm the conclusion drawn from the experiments *in vitro* that hypophysectomy and growth hormone can exert an effect on microsomes which alters their ability to synthesize protein. The depressed rate of protein biosynthesis observed in livers of hypophysectomized animals and the stimulation of protein synthesis by treatment with growth hormone (see Ketterer, Randle & Young, 1957 for review) may be at least partly accounted for by such a change in the microsomes, since it is generally accepted (see Hoagland, 1960 for review) that much protein synthesis occurs in microsomes.

The sites of protein biosynthesis in the microsomes are the ribonucleoprotein microsomal particles (Littlefield, Keller, Gross & Zamecnik, 1955; Hoagland, 1960), and it has recently proved possible to prepare these microsomal particles or ribosomes (Roberts, 1958) from rat liver in a state in which they can incorporate amino acids into protein *in vitro* (Korner, 1959*c*, 1960, 1961; Takanami, 1960; Rendi & Hultin, 1960). This paper describes the investigation of the effects of hypophysectomy of the rat and of treatment of the rat with ox growth hormone, on the incorporation of amino acids into protein of isolated rat-liver ribosomes.

## METHODS

*Animals.* Female albino rats were used. Some were hypophysectomized by the para-pharyngeal method of Ingle & Griffith (1942) and others were submitted to sham hypophysectomy. After recovery from the operation they were housed in individual cages and fed on the same ration of a powdered diet (diet 41, Bruce & Parkes, 1949) made into a thick pellet with water. The daily ration was the largest quantity of food which was entirely consumed by every rat used in the experiment (about 10 g. of diet/100 g. of rat/day), and the rats were maintained on this ration for at least 10 days before hormone treatment was begun.

*Growth hormone.* Ox growth hormone, prepared by the method of Wilhelmi, Fishman & Russell (1948) was dissolved in aq. 0·9% NaCl at pH 8·0 and diluted so that the dose could be given in 0·5 ml. of NaCl soln. by subcutaneous injection. Control rats were given 0·5 ml. of NaCl soln. in a similar manner.

*Materials.* Generally labelled L-leucine (L-[G-$^{14}$C]leucine), DL-[1-$^{14}$C]leucine, DL-[1-$^{14}$C]valine and hydrolysed algal protein labelled with $^{14}$C were obtained from The Radiochemical Centre, Amersham, Bucks. The sodium salts of ATP and GTP were obtained from Sigma Chemical Co.

*Preparation of ribosomes.* The rats were starved overnight to deplete the liver of glycogen and were killed by decapitation. The livers were removed into ice-cold 0·44 M-sucrose and were cut up into small pieces with scissors. The liver was blotted and weighed by displacement in fresh, ice-cold 0·44 M-sucrose and the same weight of liver from control and treated groups of rats was homogenized in 5 vol. of 0·44 M-sucrose in a hand-operated homogenizer (Dounce, Witter, Monty, Pate & Cottone, 1955) kept cold in an ice bath. Care was taken to use the same number of strokes of the plunger in each case so that as far as possible the tissue from every group of rats was broken to the same extent. Nuclei, debris and mitochondria were removed by centrifuging at 15 000*g* for 10 min. at 0° and most of the supernatant fluid was removed. To it was added one-ninth of its volume of freshly made, ice-cold, 5% sodium deoxycholate in 0·03 M-tris hydrochloride buffer at pH 8·2, and the ribosomes were spun down from the dissolved lipoprotein by centrifuging at 105 000*g* for 2 hr. (cf. Korner, 1961). The supernatant fluid was removed, the pellets were rinsed in 0·03 M-tris at pH 7·5 containing 5 mM-$MgCl_2$, 0·05 M-NaCl and 0·08 M-KCl (medium *A*), and the insides of the tubes wiped with tissue. The ribosomes were washed to remove last traces of deoxycholate by gentle homogenization in medium *A* and they were centrifuged again at 105 000*g*. The ribosome pellets were suspended in medium *A* or in 0·045 M-tris at pH 7·5 containing 7·5 mM-$MgCl_2$, 75 mM-NaCl and 0·12 M-KCl (medium *B*) by gentle homogenization in a small hand-operated homogenizer and made up to a known volume with the same medium.

*Preparation of microsomes, cell sap and* pH 5 *enzyme.* Rat livers were homogenized as described above and centrifuged at 15 000*g* for 10 min., and the supernatant fluid was removed and centrifuged for 2 hr. at 105 000*g* at 0°. The cell sap was removed from the microsome pellet, which was suspended in medium *B*. A pH 5 enzyme preparation (Hoagland, Keller & Zamecnik, 1956) could be made from the cell sap by drop by drop addition of cold 0·1 N-acetic acid until the pH had fallen to 5, centrifuging at 0° and dissolving the precipitate in medium *A*. Any material that did not dissolve was removed by centrifuging, and the precipitation at pH 5 and subsequent solution in medium *A* was repeated.

Great care was taken to standardize the methods of preparing ribosomes, microsomes, cell sap and pH 5 enzyme so that they could be prepared in exactly the same way from livers of differently treated groups of rats.

*Conditions of incubation.* To 0·5 ml. of ribosome or microsome suspension in medium *B* was added 0·25 ml. of cell sap in 0·44 M-sucrose. In experiments in which pH 5 enzyme dissolved in 0·25 ml. of medium *A* was used instead of cell sap in sucrose, the ribosomes and microsomes were suspended in medium *A* and not medium *B* so that the concentration of salts in the final incubation mixture was the same in both types of incubation mixture. In either case 0·25 ml. of medium *A* containing the ATP, GTP, penicillin, chloramphenicol and radioactive amino acid and in which the pH had been readjusted to 7·5 was added. Thus in each case the final composition of the 1 ml. of reaction mixture was 0·03 M-tris buffer at pH 7·5, 5 mM-$MgCl_2$, 0·05 M-NaCl, 0·08 M-KCl, 5 mM-ATP, 0·6 mM-GTP, 100 units of penicillin, 40 $\mu$g. of chloramphenicol, 0·5 $\mu$C of radioactive amino acid, about 2 mg. of ribosome protein or 4 mg. of microsome protein and about 2·5 mg. of cell sap or pH 5 enzyme protein. When cell sap was used sucrose at a concentration of 0·11 M was also present. An ATP-generating system is not necessary (Korner, 1960, 1961). The mixture was incubated with shaking at 37° for 2 hr., the reaction stopped either by the addition of 0·5 N-$HClO_4$ or by addition of ice-cold 0·44 M-sucrose containing excess of unlabelled amino acid corresponding to the radioactive one used, and the microsomes or ribosomes were reseparated from the reaction mixture by centrifuging at 105 000*g* for 2 hr. The pellet was suspended in water, and the proteins of it and of the supernatant fluid were then precipitated with 0·5 N-$HClO_4$.

*Preparation of radioactive protein for counting.* The precipitated protein was washed free from radioactive amino acids, RNA and lipids were extracted and the protein was dried as described by Korner (1959*a*). The protein was then dissolved in 0·5 ml. of A.R. formic acid and then poured on to the centre of weighed stainless-steel planchets 3 cm. in diameter. The surface of these disks is divided into five compartments by four raised concentric rings so that fluid poured into the centre distributes itself evenly over the centre compartment and then overflows into the next compartment if its volume is too great for the centre. Thus each sample is distributed over the same area. The formic acid was evaporated off under an infrared lamp, the planchets were reweighed and the radioactivity of the protein was determined with an automatic Geiger–Müller gas-flow counter. Sufficient counts were recorded to reduce the standard error to 3% or less and corrections were made for background counts. The results are expressed as the specific activity of the protein (counts/min./mg. of protein) or as counts/min./mg. of RNA.

*Statistical treatment of results.* For many of the experiments the livers of two or more rats were pooled to provide sufficient material. In each experiment replicate samples were incubated at each of the conditions studied, and the means of the results obtained with these samples were considered to constitute one experimental observation in order to overcome any variations in result that might arise by small differences in handling the material. Each experiment was repeated several times with other groups of rats kept under the same conditions, the mean of the results obtained in these separate experiments was calculated and Student's *t* test was applied to see if the difference between means was statistically significant.

*Determination of ribonucleic acid.* RNA was determined by the slight adaptation of the method of Scott, Fraccastoro & Taft (1956) that has been described by Korner (1959*a*).

*Determination of nitrogen content.* The nitrogen content of samples of the microsomes, ribosomes, cell sap, pH 5 enzyme and the residue after RNA determination was estimated by the micro-Kjeldahl technique. The value obtained was corrected for the nitrogen content of any tris or RNA present, and protein content calculated by multiplying the corrected nitrogen figure by 6·25.

## RESULTS

*Hypophysectomy and amino acid incorporation into ribosomes.* Neither ribosomes nor cell sap, when incubated without the other in the usual incubation mixture, incorporate much amino acid into protein (Table 1) but considerable incorporation occurs when both are incubated together. Table 1 shows that ribosomes and cell sap prepared from livers of hypophysectomized rats incorporate about 43% of the amount incorporated when the ribosomes and cell sap are prepared under identical conditions from normal rats. This difference is highly significant statistically ($P < 0{\cdot}001$). It is not the cell sap from livers of hypophysectomized rats that is deficient, however, for when this is

Table 1. *Incorporation of* DL-[1-$^{14}$C]*leucine into protein of ribosomes isolated from livers of normal and hypophysectomized rats*

The conditions are described in the Methods section. Results are the means of six experiments ± S.E.M.

| Source of fractions incubated | | Specific activity of ribosome protein (counts/min./mg. of protein) |
|---|---|---|
| Ribosomes | Cell sap | |
| Normal rats | — | 19 ± 3·9 |
| — | Normal rats | 6 ± 1·4* |
| Normal rats | Normal rats | 356 ± 21·9 |
| Hypophysectomized rats | Hypophysectomized rats | 152 ± 10·9 |
| Hypophysectomized rats | Normal rats | 169 ± 14·1 |
| Normal rats | Hypophysectomized rats | 329 ± 17·3 |

* Counts/min./mg. of cell-sap protein.

incubated with ribosomes from normal rat liver almost as much radioactivity is incorporated into their protein as when both parts of the system are prepared from normal rat liver. That hypophysectomy has affected the incorporating ability of the ribosomes is confirmed (Table 1) by the observation that ribosomes from hypophysectomized rats incorporate less than those from normal liver even if cell sap from normal rat liver is used in place of that from hypophysectomized rat liver.

Very similar results to those shown in Table 1 were obtained in experiments in which a pH 5 enzyme preparation was used in place of cell sap as a source of amino acid-activating enzymes (Hoagland *et al.* 1956) and soluble RNA (Hoagland, Zamecnik & Stephenson, 1957).

No consistent differences in RNA:protein ratio of ribosomes from normal and hypophysectomized rats was noted, consequently the differences in incorporation between them which have been found hold even if they are calculated as counts/min./mg. of RNA.

*Growth-hormone treatment of rats and amino acid incorporation into ribosomes.* Groups of hypophysectomized and normal rats were given various doses of ox growth hormone by subcutaneous injection for 5 days and the incorporation *in vitro* of amino acid into protein of ribosomes isolated from their livers was measured. The results (Table 2) show that treatment of hypophysectomized rats with growth hormone could restore the incorporation obtained almost to normal levels and that greater than normal levels of incorporation were found on treatment of normal rats with growth hormone. Experiments of the type depicted in Table 1 showed that most of the change brought about by treatment of rats with ox growth hormone could be accounted for by the increased ability of the ribosomes to incorporate amino acids into protein. Little change in the ability of the cell sap to prepare amino acids for incorporation was detected.

*Effect of hypophysectomy on the stability of ribosomes.* Korner (1961) has shown that ribosomes can be preincubated at 37° in medium *A* for about 1 hr. without substantial fall in their ability to incorporate amino acids into protein. It is possible that hypophysectomy of the rat may have altered the liver ribosomes in such a way that they are less stable than those from normal rat liver. The differences in amino acid incorporation found could then be explained by differences in the stability of the ribosomes. An examination of the increase in ribosome labelling with time of incubation (Fig. 1) does not, however, support this suggestion. Ribosomes from liver of hypophysectomized rats become radioactive more slowly than those of normal rats right from the beginning of incubation, and increases in the specific activity of ribosome protein in the preparation from hypo-

Table 2. *Incorporation of* DL-[1-$^{14}$C]*leucine into protein of ribosomes isolated from livers of normal and hypophysectomized rats injected with ox growth hormone*

| | Growth hormone given each day for 5 days (mg.) | Counts/min./mg. of ribosome protein |
|---|---|---|
| Normal rats | 0 | 348±29 |
| | 0·01 | 391±22 |
| | 0·25 | 407±19 |
| | 1·0 | 401±31 |
| Hypophysectomized rats | 0 | 172±22 |
| | 0·01 | 249±14 |
| | 0·25 | 283±17 |
| | 1·0 | 298±24 |

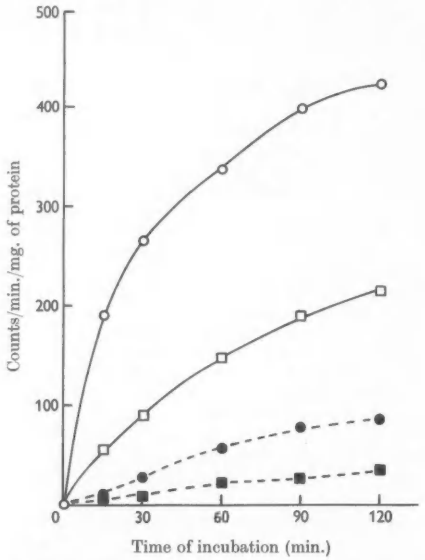


Fig. 1. Time-course of incorporation of L-[G-$^{14}$C]leucine into protein of ribosomes and cell sap isolated from livers of normal and hypophysectomized rats. Incubation of ribosomes and cell sap from normal rat liver and from hypophysectomized rat liver was carried out under conditions described in the text. At the end of incubation ribosomes and cell sap were separated from each other as described in the Methods section and the radioactivity of the proteins of each was assessed. ○, Ribosomes from normal rats; □, ribosomes from hypophysectomized rats; ●, cell sap from normal rats; ■, cell sap from hypophysectomized rats.

physectomized rats keep more or less in step with increases in specific activity obtained with ribosomes from normal rat liver.

The results in Table 3 offer support for the contention that no difference is apparent in the stability of ribosomes as a result of hypophysectomy or treatment with growth hormone, for little difference in the ability of ribosomes from the differently treated groups of rats to withstand preincubation was detected.

*Transfer of protein to supernatant fluid.* When ribosomes are incubated, radioactive protein appears in the supernatant fluid, and it has been suggested, but not proved, that this occurs by transfer of labelled protein from ribosomes to supernatant rather than by breakdown of ribosomes (Korner, 1961). Similar percentages of the total counts in the incubation mixture appeared in non-ribosomal protein at various times during an experiment whether the ribosomes came from livers of normal rats or from livers of hypophysectomized rats. This conclusion is illustrated in Fig. 1, which shows that the relationship of specific activity of the cell-sap protein to that of the ribosome protein at different times of incubation is similar in the systems prepared from normal and hypophysectomized rat liver.

Table 3. *Effect of preincubation of ribosomes from livers of normal and hypophysectomized rats and from rats treated with growth hormone on their subsequent ability to incorporate radioactive amino acids into protein*

Incorporation at zero time was taken as 100.

| Time of preincubation (min.) | Ribosomes from: Normal rats | Ribosomes from: Hypophysectomized rats |
|---|---|---|
| 0 | 100 | 100 |
| 15 | 96 | 93 |
| 30 | 90 | 91 |
| 60 | 81 | 78 |

*Magnesium and adenosine triphosphate concentration on amino acid incorporation into ribosomes from normal and hypophysectomized rat liver.* The concentration of magnesium ions and of ATP and the relative proportions of them are critical variables in controlling the amount of amino acid which is incorporated into ribosomes (Korner, 1961). Incorporation of amino acid into protein of ribosomes from hypophysectomized rats showed the same behaviour towards these variables as the ribosomes from normal rat liver. The best incorporation was seen at 0·01 M-ATP and 0·01 M-$MgCl_2$ in both cases (cf. Korner, 1961). Many other concentrations of magnesium and ATP were tested and these caused large differences in the incorporation obtained, but in each case the incorporation of amino acids into ribosomes from normal rat liver was always greater than that into ribosomes from hypophysectomized rat liver.

*Comparison of the effects of hypophysectomy on microsomes and on ribosomes.* A group of rats were hypophysectomized and they, and some sham-operated control rats, were treated with various doses of ox growth hormone. Homogenates of liver of each group of rats were made in the usual way, and both microsomes and ribosomes were prepared from the same homogenate. The extent of incorporation of amino acids into protein in the microsomes and ribosomes was compared under standard conditions with the same pH 5 enzyme preparation in each case. Table 4 shows that, when compared with untreated control rats, hypophysectomy or growth-hormone treatment produced broadly similar percentage changes in specific activity of protein of ribosomes and of microsomes.

## DISCUSSION

The experiments reported in this paper support the idea that hypophysectomy of the rat changes the liver ribosomes in a way that makes them less capable of synthesizing protein than those of normal rat liver and that treatment of rats with ox growth

Table 4. *Incorporation of* DL-[$^{14}$C]*valine into protein of ribosomes and microsomes from livers of normal and hypophysectomized rats and rats treated with ox growth hormone*

| | Growth hormone given each day for 5 days (mg.) | Counts/min./mg. of microsome protein | Change compared with untreated normal rats (%) | Counts/min./mg. of ribosome protein | Change compared with untreated normal rats (%) |
|---|---|---|---|---|---|
| Normal rats | 0 | 168 | — | 404 | — |
| | 0·01 | 198 | 17·9 | 482 | 19·3 |
| | 1·0 | 217 | 29·2 | 507 | 25·3 |
| Hypophysectomized rats | 0 | 70 | −58·4 | 157 | −61·0 |
| | 0·01 | 119 | −29·2 | 317 | −21·5 |
| | 1·0 | 135 | −19·7 | 351 | −13·1 |

hormone causes changes in ribosomes which enhance their protein-synthesizing ability. Little change was detected in the activity of the amino acid-activating enzymes (Hoagland *et al.* 1956) or in the ability of the soluble RNA (Hoagland *et al.* 1957) to form a complex with amino acids and to transfer it to ribosomes.

The possibility that the changes in incorporation of amino acids into protein resulting from hypophysectomy were brought about by altered stability of the ribosomes or by changes in their requirements for incorporation was ruled out by some of the experiments described (Fig. 1; Table 3). Support for the idea that growth hormone affects ribosomes *in vivo* in a way that enables them to incorporate more amino acids in the *in vitro* system is provided by these experiments and this suggestion is reinforced by the results of experiments with whole animals (Korner, 1959*b*).

The changes that are known to occur in the amino acid-incorporating ability of microsomes as a result of hypophysectomy of rats or treatment of rats with growth hormone (Korner, 1959*a*) can be explained by changes in the ribosome part of them, for the percentage change in incorporation after hypophysectomy and growth-hormone administration was the same in ribosomes as in microsomes.

Tne change in ribosomes brought about by growth-hormone treatment or hypophysectomy of the rat does not appear to alter the ability of ribosomes to release labelled protein to the supernatant fluid (Fig. 1), so the change must have occurred in the ability of the ribosome to accept activated amino acids and to link them in polypeptide chains.

Little or no difference in RNA:protein ratio was detected between ribosomes from livers of normal and hypophysectomized rats. Perhaps there is less non-active RNA in ribosomes from liver of rats that have circulating growth hormone or there may be less inactive ribosomes. It has been suggested for at least one system (Tissière, Schlessinger & Gros, 1960) that only a very small proportion of the total ribosomes of the cell are able to incorporate amino acids into protein; it is possible that the number that are active is controlled in some way by growth hormone.

The results reported in this paper offer an at least partial explanation of the known effects of hypophysectomy and of growth-hormone treatment on body weight, carcass protein, nitrogen retention and protein biosynthesis (Lee & Schaffer, 1934; Lee & Ayres, 1936; Simpson, Evans & Li, 1949; Russell, 1951, 1955; Ketterer *et al.* 1957), in terms of hormonally-induced changes of the templates on which protein synthesis occurs (Littlefield *et al.* 1955; Hoagland, 1960).

## SUMMARY

1. Hypophysectomy of the rat reduces, and treatment of normal and of hypophysectomized rats with ox growth hormone increases, the incorporation of radioactive amino acids into protein of isolated liver ribosomes.

2. The ribosomes from hypophysectomized rat liver were no less stable than those from normal rat liver, nor did their requirements for incorporation differ significantly from those of normal rat liver.

3. A similar proportion of labelled protein was transferred to the supernatant fluid from ribosomes of liver of hypophysectomized rats as from those of normal rats.

4. The effects of hypophysectomy of the rat and treatment with growth hormone on microsomes that have been previously demonstrated (Korner, 1959*b*) can be explained in terms of the hormonal effects on the ribosome part of the microsomes.

5. It is concluded that the effects of growth hormone on protein biosynthesis in rats can be explained at least partly in terms of hormonally induced changes of the ribosomes in which protein synthesis takes place.

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# The Inhibition of Acetylcholine Synthesis in Brain by a Hemicholinium

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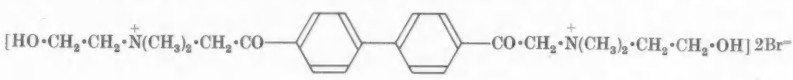
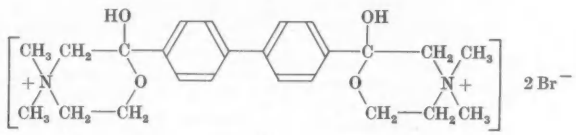
Long & Schueler (1954) described a series of bisquaternary ammonium compounds based on $\alpha\alpha'$-dibromo-4:4-bisacetophenone including one, obtained by treating the dibromo compound with dimethylaminoethanol, which was formulated as in Fig. 1*a*. As it was extremely toxic when injected into animals but possessed only weak anticholinesterase activity, and as the infrared spectrum showed strong bands due to ether groups, Schueler (1955) suggested the hemiacetal formulation (Fig. 1*b*) and described the compound as a 'hemicholinium'. The compound of Fig. 1 is usually referred to as HC3.

Long & Schueler found that, when injected into animals, HC3 paralysed the respiration; the paralysis developed after some delay and was reversed by injecting choline or anticholinesterases. MacIntosh, Birks & Sastry (1956) found that low concentrations of HC3 (0·01–0·1 mM) inhibited the synthesis of acetylcholine in the perfused superior cervical ganglion of the cat and in minced brain of the mouse; the inhibition was reversed by adding choline in similar concentrations. MacIntosh *et al.* (1956) therefore suggested that HC3 somehow inhibited the formation of acetylcholine. However, choline acetylase extracted from acetone-dried powder of brain was hardly inhibited at all by HC3, even when the concentration of HC3 was one thousand times that of choline. This made it unlikely that the inhibition of acetylcholine formation thought to occur *in vivo* was due to a direct effect of HC3 on choline acetylase.

The possibility remained, however, that the absence of inhibition *in vitro* was either because the enzyme was changed during its extraction or because HC3 competed with choline for access to the enzyme in the cell. These alternatives had to be examined because in the brain 75 % or more of the choline acetylase is associated with intracellular particulate fractions that are fully active only after




(*a*)




(*b*)

Fig. 1

they have been treated with an organic solvent such as ether. This suggests that a structural barrier has to be disrupted before the enzyme is fully active. HC3 may therefore act by competing with choline in its movement through such a barrier. This paper shows the effect that HC3 has upon the choline-acetylase activity of particles obtained from guinea-pig brain. When the choline concentration is low HC3 inhibits the enzyme as long as the particles are intact but not after they have been treated with ether. A preliminary account of this work has been given (Gardiner, 1957).

## MATERIALS AND METHODS

*Abbreviation.* TEPP, Tetraethyl pyrophosphate.

*HC3.* This was synthesized as described by Long & Schueler (1954) (Found: Br, 27·0. Calc.: Br, 27·6%). The u.v.-absorption spectrum and the chemical properties were as described by Schueler (1955).

*Pigeon-liver extract.* This was prepared according to Kaplan & Lipmann (1948).

*Acetyl-coenzyme A.* This was prepared from coenzyme A (Pabst Laboratories) by acetylation with acetic anhydride (Stadtman, 1957).

*Tetraethyl pyrophosphate.* A commercial sample (L. Light and Co. Ltd.) was redistilled under reduced pressure with appropriate precautions (Toy, 1948). The fraction boiling at 96–98° under 0·05–0·1 mm. Hg was collected and kept in 0·5 ml. portions in sealed ampoules. After the ampoules were opened they were kept in a stoppered tube and discarded after a fortnight. Solutions of TEPP were made up in 0·01 M-potassium phosphate buffer, pH 7·4, immediately before use.

*Acetylcholine.* This was estimated pharmacologically on the isolated frog rectus abdominis with acetylcholine perchlorate (British Drug Houses Ltd.) as standard. The muscle was sensitized towards acetylcholine by treatment with TEPP (Hobbiger, 1950). The semi-automatic device described by Boura, Mongar & Schild (1954) was used; the samples were added manually at the appropriate times.

The solutions to be assayed contained substances other than acetylcholine which affected the frog muscle. Their effect was compensated for by adopting the method of Feldberg (1950). The comparison solution was freed from acetylcholine as follows: one drop of Universal Indicator (British Drug Houses Ltd.) was added to 2 ml. of the solution to be assayed, NaOH (0·33 N) was added dropwise until the colour changed to purple, and one more drop was added. The alkaline solution was heated in a stoppered test tube for 2 min. in boiling water, chilled in ice–water and neutralized with HCl (0·33 N). The volume was made up to 4 ml. with frog Ringer solution. This treatment was sufficient to destroy the acetylcholine; greater alkalinity changed the sucrose or HC3, or both, in the solution so that they affected the frog rectus.

With small quantities of acetylcholine, or for insensitive preparations of the muscle, an approximately fivefold increase in sensitivity could be obtained by adding a potentiating mixture. This contained M-potassium phosphate buffer, pH 7·4 (1 ml.), 0·2 M-choline chloride (0·5 ml.) and frog Ringer solution to 25 ml. Since this mixture by itself made the rectus contract the maximum amount that could be added was four-fifths of the dose that produced a just perceptible contraction. The same amount was added to all test solutions by means of a syringe arranged to deliver a pre-set volume.

*Mouse- and guinea-pig-brain minces.* The animals were killed by decapitation and the brains were removed and chilled. The cold tissue was placed in a glass tube closed at one end by a close-fitting plunger and at the other by two layers of fine nylon stocking. The mince was prepared by extruding the tissue slowly through the nylon mesh; as it emerged it was scraped off and transferred to a chilled weighed flask. The mince was suspended in a modified Locke's solution containing bicarbonate (Mann, Tennenbaum & Quastel, 1939), and samples, with the appropriate additions of HC3, were incubated in Warburg manometers. The incubated mixtures were prepared for the assay of acetylcholine as described by Mann *et al.*

*Guinea-pig-brain suspension.* The brains of two or more animals were removed as described and ground in an all-glass homogenizer in 9 vol. of ice-cold 9% (w/v) sucrose solution. The sucrose solution contained ethylenediaminetetra-acetic acid (1·0 mM) and was adjusted to pH 7·4 with NaOH. The tissue suspension was centrifuged at 1000*g* for 10 min. at 0–1° to remove intact cells and nuclei. The supernatant was a cell-free suspension which contained the choline acetylase. The suspension was used as such, or it was centrifuged at 15 000*g* for 10 min. to sediment the mitochondrial fraction (Gardiner, 1959). This was resuspended in sucrose solution. It contained less soluble choline acetylase, and also less choline.

Each enzyme preparation was divided into two parts. One part was stored in ice–water for no longer than 1 hr. before use. The other was treated with $\frac{1}{5}$ vol. of peroxide-free ether and kept for 20–30 min. in ice–water. The ether was removed by blowing $N_2$ through the suspension just before use; frothing was prevented with silicone anti-foam (Midland Silicones; type A).

*Choline-acetylase assays.* The reaction was carried out in stoppered graduated test tubes, which had been selected to withstand centrifuging. The reaction mixtures were kept ice-cold until all components had been added; they were then incubated at 37° with gentle shaking. To stop the reaction, enough acid was added to make the pH 3–4, and the tubes were heated for 5 min. in boiling water. After cooling thoroughly in ice–water the mixture was neutralized with NaOH, made up to 5 ml. with frog Ringer solution and centrifuged. The supernatant, which contained the acetylcholine, was frozen and stored at −20° until assay.

## RESULTS

*Minced mouse and guinea-pig brain.* MacIntosh *et al.* (1956) found that when HC3 was added to minced mouse brain it inhibited the formation of acetylcholine. This was confirmed; the inhibition was less than they had found, but so also was the synthetic activity. HC3 also inhibited the synthesis of acetylcholine in minced guinea-pig brain; Table 1 shows that HC3 (0·1 mM) reduced the synthesis by up to four-fifths in guinea-pig brain and by only two-thirds in mouse brain.

*Cell-free homogenates of brain.* The effect of HC3 was tested on the synthesis of acetylcholine

by cell-free homogenates of brain prepared as described in the Materials and Methods section. The work of MacIntosh *et al.* (1956) had suggested that HC3 would inhibit the synthesis of acetylcholine only if the concentration of choline was much below that usually used for determining the activity of the synthesizing enzymes. This was confirmed. Table 2 shows that HC3 inhibited the formation of acetylcholine only when the concentration of added choline was less than 25 $\mu$M.

Under these conditions the amount of acetylcholine synthesized was small and variable. Hebb (1955) suggested that this might be due to an inadequate supply of acetyl-coenzyme A. The brain suspension was therefore fortified with pigeon-liver extract and citrate. Table 3 shows that when this was done more acetylcholine was formed even in the absence of added choline, and in the presence of 0·1 mM-HC3 this synthesis was reduced by 40 %. When 25 $\mu$M-choline was added the synthesis was reduced by only 14 %.

*Particulate choline acetylase of guinea-pig brain.* To obtain information about the site at which HC3 inhibited acetylcholine formation it was necessary to simplify the system, for the following reasons: Crude homogenates of brain contain choline acetylase in at least two forms (Hebb & Smallman, 1956). About 25 % of the total activity is soluble, and there is evidence that soluble choline acetylase is not inhibited by HC3 (MacIntosh *et al.* 1956). As long therefore as soluble choline acetylase was present, its activity might have masked any inhibition by HC3 of insoluble choline acetylase. Further, it was desirable to reduce the concentration of endogenous choline.

For these reasons, the crude homogenates were used to prepare mitochondrial fractions, which Hebb & Smallman (1956) had shown to contain most of the choline-acetylating activity.

Hebb (1955) and Reisberg (1957) reported that choline acetylase was inhibited by excess of acetyl-coenzyme A and that the rate of acetylcholine synthesis was greater if the precursors of acetyl-coenzyme A were present rather than acetyl-coenzyme A itself. However, the mitochondrial fraction of brain synthesized moderate amounts of acetylcholine when incubated with choline and acetyl-coenzyme A itself. Fig. 2 shows that, in the presence of 10 mM-choline, as the concentration of acetyl-coenzyme A was increased so was the

Table 1. *Inhibition of acetylcholine synthesis in minced brain by HC3* (0·1 *mM*)

The flasks contained 150 mg. wet wt. of brain mince, 0·5 mg. of eserine sulphate, where indicated 0·32 $\mu$mole of HC3, and bicarbonate–Locke's solution to 3·2 ml. They were incubated, with gentle shaking, in an atmosphere of $O_2 + CO_2$ (95:5) for the time stated below.

| Animal | Incubation (hr.) | Acetylcholine formed ($\mu$m-moles/g. wet wt.) Without HC3 | With HC3 | Inhibition by HC3 (%) |
|---|---|---|---|---|
| Mouse | 3½ | 39 | 12 | 69 |
| Guinea pig | 4½ | 54 | 9 | 83 |
| Guinea pig | 1½ | 39 | 10 | 74 |
| Guinea pig | 1¼ | 41 | 8·7 | 79 |

Table 2. *Inhibition of synthesis of acetylcholine by HC3 in cell-free homogenates of guinea-pig brain*

Reaction mixture contained in 2 ml.: 1 ml. of brain suspension, 0·8 ml. of substrate mixture, choline chloride solution, HC3 solution, or water as appropriate. Substrate mixture contained in 8·5 ml.: 1 ml. of M-potassium phosphate buffer, pH 7·4, 1 $\mu$mole of coenzyme A, 0·1 m-mole of adenosine triphosphate, 0·2 m-mole of sodium acetate, 0·1 m-mole of L-cysteine hydrochloride, 0·4 m-mole of $MgCl_2$, 20 $\mu$moles of TEPP. Incubation was for 1 hr. at 37°. Results are corrected for acetylcholine content of homogenate and synthesis from endogenous substrates.

| No. of experiments | Concn. of HC3 ($\mu$M) | Concn. of added choline ($\mu$M) | Net acetylcholine formed ($\mu$m-moles/g. wet wt.) | Inhibition (%) |
|---|---|---|---|---|
| 1 | 0 | 10 000 | 330 | — |
| | 50 | 10 000 | 330 | 0 |
| 2 | 0 | 50 | 7 | — |
| | 50 | 50 | 7 | 0 |
| 1 | 0 | 25 | 14 | — |
| | 50 | 25 | 12 | 14 |
| 1 | 0 | None | 12 | — |
| | 50 | None | 3 | 75 |

Table 3. *Inhibition of acetylcholine synthesis by HC3 in cell-free homogenate of guinea-pig brain with augmented acetyl-coenzyme A synthesis*

The reaction mixtures contained in 2 ml.: 1 ml. of brain suspension, 2 $\mu$moles of TEPP, 0·7 ml. of substrate mixture, choline chloride solution, HC3 solution, or water as appropriate. The substrate mixture contained in 7·4 ml.: 2 ml. of 'aged' pigeon-liver extract, 0·5 ml. of M-potassium phosphate buffer, pH 7·4, 0·3 m-mole of citric acid, 0·4 m-mole of sodium acetate, 0·15 m-mole of L-cysteine hydrochloride, 0·1 m-mole of adenosine triphosphate, 1 $\mu$mole of coenzyme A, 5N-KOH to adjust pH to 7·4, water to volume. Incubation was for 1 hr. at 37°. Results are corrected for acetylcholine content of homogenate and synthesis from endogenous reactants.

| No. of experiments | Concn. of HC3 ($\mu$M) | Concn. of added choline ($\mu$M) | Average synthesis of acetylcholine ($\mu$m-moles/g. wet wt.) | Activation (+) or inhibition (−) (%) |
|---|---|---|---|---|
| | | Untreated homogenate | | |
| 5 | 0 | 25 | 34 | — |
| | 100 | 25 | 26 | −14 |
| 5 | 0 | None | 25 | — |
| | 100 | None | 15 | −40 |
| | | Ether-treated homogenate | | |
| 5 | 0 | 25 | 60 | — |
| | 100 | 25 | 73 | +22 |
| 5 | 0 | None | 43 | — |
| | 100 | None | 59 | +37 |

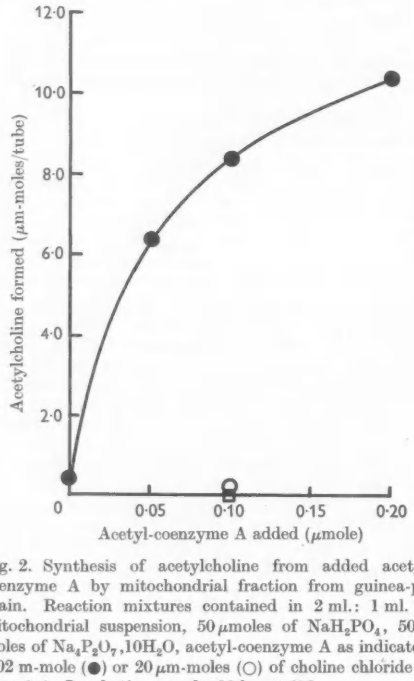



Fig. 2. Synthesis of acetylcholine from added acetyl-coenzyme A by mitochondrial fraction from guinea-pig brain. Reaction mixtures contained in 2 ml.: 1 ml. of mitochondrial suspension, 50 $\mu$moles of $NaH_2PO_4$, 50 $\mu$moles of $Na_4P_2O_7$,$10H_2O$, acetyl-coenzyme A as indicated, 0·02 m-mole (●) or 20 $\mu$m-moles (○) of choline chloride or none (□). Incubation was for 1¼ hr. at 37°.

amount of acetylcholine formed. Acetyl-coenzyme A (0·1 $\mu$mole) was used in subsequent experiments. When no choline was added no acetylcholine was formed. Thus the enzyme activity was limited by lack of choline rather than by excess of added acetyl-coenzyme A. When 20 $\mu$m-moles of choline was added some acetylcholine was formed, but even with so little choline the proportion of it that was acetylated was small.

The conditions so established were used for determining the effect of HC3 on the synthesis of acetylcholine by mitochondrial fractions of brain. Table 4 shows that when the concentration of added choline was 25 $\mu$M, HC3 at a concentration of 0·1 mM inhibited acetylcholine synthesis by nearly 50%.

*Effect of varying the concentrations of choline and HC3 on the synthesis of acetylcholine by brain particles.* In two experiments higher concentrations of both choline and HC3 were used; the results are in Table 5. When the concentration of HC3 only was increased the inhibition of acetylcholine synthesis was increased. However, if the choline concentration was also increased inhibition by HC3 was diminished. The effect of increasing the concentration of choline was not offset by increasing that of HC3.

*Acetylcholine synthesis from endogenous reactants.* When a homogenate of guinea-pig brain was incubated with an anticholinesterase but without added substrates a little acetylcholine was syn-

thesized. Table 6 shows that this synthesis was not affected by HC3. When the mitochondrial fraction was incubated in this way traces of acetylcholine were usually formed but again HC3 had no effect.

*Cell-free suspension of guinea-pig brain after treatment with ether.* Samples of the brain homogenates were treated with ether (see Methods) and the effect of HC3 on acetylcholine synthesis was determined. The results in Table 3 show that more acetylcholine was produced when homogenates had been treated with ether than when not, and the synthesis by ether-treated homogenates was not inhibited by HC3. On the contrary, in the presence of HC3 the formation of acetylcholine was usually increased. The average increase produced by 0·1 mM-HC3 was 37% when no choline was added and 22% when choline was added at a concentration of 25 $\mu$M.

*Mitochondrial fraction after ether treatment.* The effect of HC3 on mitochondrial fractions that had been treated with ether was similar to that on the homogenates. Table 4 shows that the choline acetylase was between two and four times as active and, again, HC3 (0·1 mM) produced not inhibition but activation. The effect was less than with the homogenates for the average activation was only 10%.

Table 4. *Inhibition by HC3 of acetylcholine synthesis by mitochondrial fraction of guinea-pig brain with added acetyl-coenzyme A*

The reaction mixtures contained in 2 ml.: 0·1 $\mu$mole of acetyl-coenzyme A, 2 $\mu$moles of TEPP, 50 $\mu$moles of $NaH_2PO_4$, 50 $\mu$moles of $Na_4P_2O_7$,$10H_2O$, 0·05 $\mu$mole of choline chloride, 1 ml. of suspension of mitochondrial fraction of brain and 0·2 $\mu$mole of HC3 if present. Incubation was for 1½ hr. at 37°. The results are corrected for acetylcholine content of particles and for any synthesized from endogenous choline.

| Prep. no. | Acetylcholine formed ($\mu$m-moles/ml. of suspension) Without HC3 | With HC3 | Activation (+) or inhibition (−) (%) |
|---|---|---|---|
| | Untreated mitochondrial fraction | | |
| 1 | 3·3 | 2·5 | −25 |
| 2 | 5·2 | 2·6 | −50 |
| 3 | 3·3 | 1·9 | −43 |
| 4 | 2·5 | 1·0 | −60 |
| 5 | 5·3 | 2·5 | −53 |
| | Ether-treated mitochondrial fraction | | |
| 1 | 7·0 | 9·0 | +28 |
| 2 | 16·0 | 16·0 | Nil |
| 3 | 7·0 | 7·1 | + 1 |
| 4 | 9·4 | 10·4 | +11 |
| 5 | 16·3 | 18·2 | +12 |

## DISCUSSION

The results have shown that the synthesis of acetylcholine by intracellular particles obtained from brain was inhibited by HC3, but not if the particles were first treated with ether. Treatment with ether is thought to disrupt the membrane which encloses intracellular particles. The observations suggest, therefore, that HC3 inhibits not by a direct effect on choline acetylase but by an indirect effect which involves the integrity of the particles.

It was found, further, that the degree of inhibition depended, amongst other things, upon the concentration of choline in the medium: as the concentration of choline was reduced below about

Table 5. *Inhibition of acetylcholine synthesis by HC3: effect of various concentrations of choline and HC3*

The reaction mixtures contained in 2 ml.: 0·1 $\mu$mole of acetyl-coenzyme A, 2 $\mu$moles of TEPP, 50 $\mu$moles of $NaH_2PO_4$, 50 $\mu$moles of $Na_4P_2O_7$,$10H_2O$, 1 ml. of suspension of mitochondrial fraction of brain. Other additions were as listed below. Incubation was for 1½ hr. at 37°.

| Choline added ($\mu$mole) | 0 | 0·05 | 0·1 | 0·2 | 0 | 0·05 | 0·1 | 0·2 | 0 | 0·05 | 0·1 | 0·2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HC3 added ($\mu$mole) | 0 | | | | 0·2 | | | | 0·4 | | | |
| Assay results ($\mu$m-moles of acetylcholine/ml. of suspension) | 1·5 | 3·9 | 6·0 | 8·0 | 1·5 | 2·8 | 5·8 | 8·0 | 1·5 | 2·3 | 5·3 | 7·0 |
| Results corrected for acetylcholine content of enzyme suspension | 0·5 | 2·9 | 5·0 | 7·0 | 0·5 | 1·8 | 4·8 | 7·0 | 0·5 | 1·3 | 4·3 | 6·0 |
| Results corrected for synthesis from endogenous choline | — | 2·4 | 4·5 | 6·5 | — | 1·3 | 4·3 | 6·5 | — | 0·8 | 3·8 | 5·5 |
| Inhibition (%) | — | — | — | — | — | 48 | 5 | 0 | — | 68 | 12 | 15 |
| Assay results ($\mu$m-moles of acetylcholine/ml. of suspension) | 2·2 | 4·2 | 8·8 | 9·7 | 2·2 | 3·0 | 8·5 | 9·3 | 2·2 | 2·6 | 7·9 | 9·3 |
| Results corrected for acetylcholine content of enzyme suspension | 0 | 2·0 | 6·6 | 7·5 | 0 | 0·8 | 6·3 | 7·1 | 0 | 0·4 | 5·7 | 7·1 |
| Results corrected for synthesis from endogenous choline | — | 2·0 | 6·6 | 7·5 | — | 0·8 | 6·3 | 7·1 | — | 0·4 | 5·7 | 7·1 |
| Inhibition (%) | — | — | — | — | — | 60 | 5 | 5 | — | 80 | 14 | 5 |

Table 6. *Synthesis of acetylcholine from endogenous reactants in the presence and the absence of HC3* (0·1 mM)

Mixtures contained in 2·2 ml.: 2 ml. of homogenate or suspension of mitochondrial fraction of guinea-pig brain, 2 μmoles of TEPP, and, if present, 0·2 μmole of HC3. Incubation was as in previous experiments.

| Enzyme prep. | Acetylcholine formed (μm-mole/ml. of suspension) | |
|---|---|---|
| | Without HC3 | With HC3 |
| Cell-free homogenate | 1·1 | 1·1 |
| Mitochondrial fraction | 0·35 | 0·35 |

50 μM the inhibition produced by a given concentration of HC3 increased. The results suggest the possibility that HC3 inhibits acetylcholine synthesis by competing with choline for access to the enzyme. Putting the observations together, it is a reasonable conclusion that HC3 is able to block the passage of choline across the membrane enclosing the particles.

Hebb & Smallman in their studies on the intracellular distribution of choline acetylase considered the possibility that the enzyme was enclosed within a barrier or membrane. More recent work (Hebb & Whittaker, 1958; Bellamy, 1959; Whittaker, 1959) supports this idea by showing that both choline acetylase and acetylcholine are associated with particles that have sedimentation characteristics similar to those of mitochondria but which may be distinguished from them. Whittaker (1959) has shown that organic solvents will damage these particles to such an extent that they no longer retain acetylcholine. By milder treatment only some of the acetylcholine is released but a barrier remains which prevents its return. It seems likely that this is the membrane through which the passage of choline is restricted and at which HC3 competes with it.

Such a barrier to the entry of choline would explain why acetylcholine is synthesized so slowly in intact particles. The existence of the barrier might also explain why HC3 has no effect on the very slight endogenous synthesis of acetylcholine, because this is presumably made from choline that has already penetrated into the particles and is free inside them. MacIntosh, Birks & Sastry (1958) have provided other evidence that HC3 affects a transport mechanism for choline. They have shown that in the chicken HC3 inhibits the excretion of choline by the kidney, a function that does not involve choline acetylase.

The present results do not support the possibility that HC3 acts by inhibiting choline acetylase directly, nor that there is no inhibition after treatment with ether because the enzyme itself is changed in some way. If this were so one would expect that the inhibition would increase as the structure of the tissue was broken down, since both choline and HC3 should have easier access to the enzyme; in fact, the inhibition decreased.

The results obtained with other preparations support the conclusion that the effectiveness of HC3 depends upon the structural integrity of tissues. Thus in the whole animal HC3 is extremely toxic. In intact nervous tissue, i.e. in the superior cervical ganglion of the cat, perfusing with low concentrations of HC3 (0·01–0·1 mM) inhibits the production of acetylcholine. In minced brain, in which the nerve cells are still largely intact, HC3 inhibits but less strongly. When the cells in turn are disrupted HC3 still inhibits the synthesis of acetylcholine that proceeds in intracellular particles. When finally these particles are broken down by ether, HC3 inhibits no longer; nor does it inhibit choline acetylase extracted from acetone-dried powders of brain, in which all structure has presumably been destroyed. It seems, then, that HC3 inhibits the activity of choline-acetylating systems by competing with choline for passage through membranes.

An unexpected observation was that the amount of acetylcholine formed by ether-treated preparations acting upon low concentration of choline increased when HC3 was present. This increase was not reported by MacIntosh *et al.* (1956), who used soluble choline acetylase. It seems, therefore, that activation by HC3, like inhibition, was related to the complexity of the enzyme preparation, in that activation was greatest with the homogenates and less with the particles; it is apparently absent with extracts of acetone-dried powder. Even the most active preparations acetylated only 30–40 % of the added choline. Therefore, one reason for the activation by HC3 may be that it inhibits other enzymes utilizing choline and that, because of this, more of the limited amount of choline present is available for acetylation.

In the whole animal HC3 is very toxic, and the problem remains whether the toxicity may be explained by the inhibition of acetylcholine synthesis. The experiments described in this paper offer little support for this idea in that the concentrations of HC3 needed to inhibit acetylcholine synthesis were much higher than the concentrations which are toxic *in vivo*. Thus if the $LD_{50}$ for a guinea pig were uniformly distributed throughout the animal the concentration would be 0·1 μM, and, if throughout the extracellular fluid, 0·5 μM. Against that the concentration required *in vitro* to inhibit brain choline acetylase by 50 % was 0·1 mM. The discrepancy between the concentrations of HC3 required to inhibit choline acetylation *in vitro* and those toxic to the whole animal may be

explained by a readier access of choline to the enzyme *in vitro*.

It has always been difficult to accept that the toxicity of HC3 is due to effects on the central nervous system (Schueler, 1955; Kasé & Borison, 1958) because it is unlikely that a bisquaternary ammonium compound of this type would penetrate sufficiently. Recently, evidence has appeared suggesting that HC3 interrupts neuromuscular transmission in the respiratory musculature (Longo, 1959; Holmes & Wilson, 1960). This effect appears only when the rate of stimulation is high and sustained; this would deplete the acetylcholine in the nerve endings and would aggravate any limitation on its resynthesis. Moreover, the effect was rapidly and readily reversed by choline, which is in keeping with the nature of the inhibition found in the present work.

## SUMMARY

1. The inhibition by HC3 of acetylcholine synthesis in minced mouse brain has been confirmed. The inhibition was observed also in minced guinea-pig brain.

2. The synthesis of acetylcholine by homogenates of guinea-pig brain or by particles obtained from them was inhibited by HC3 only if the concentration of choline was low.

3. When these preparations were treated with ether the increased synthesis of acetylcholine that occurred was activated by HC3. This effect was more marked with the cruder preparations.

4. It was concluded that HC3 does not inhibit choline acetylase directly but that it competes with choline for access to the enzyme through structural barriers in the cell.

5. The relation of these findings *in vitro* to the high toxicity of HC3 is discussed.

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# Incorporation of [2-$^{14}$C]Mevalonic Acid and [2-$^{14}$C]Acetic Acid into Lipids of Mycobacteria

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The present investigation was started with a view to studying the biosynthesis of vitamin $K_2$, which is known to occur in mycobacteria (Snow, 1952; Brodie, Davies & Fieser, 1958). Vitamin $K_2$ isolated from *Mycobacterium tuberculosis* (Brevannes) has nine isoprene units in its side chain (Noll, 1958; Noll, Ruegg, Gloor, Ryser & Isler, 1960). Evidence has been presented by Brodie & Ballantine (1960) that vitamin K has an essential role in electron transport and oxidative phosphorylation in preparations from mycobacteria. It is known that animal tissues do not synthesize

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vitamin K. Hence, an understanding of the biosynthetic pathway of vitamin $K_2$ in *M. tuberculosis* might offer a possibility of finding a suitable inhibitor of one of the steps and thereby act as a therapeutic agent.

[2-$^{14}$C]Mevalonic acid, the recognized precursor of isoprenoid compounds in Nature, was found to be incorporated into the lipids of *M. tuberculosis*. Since this organism lacks the other generally occurring isoprenoids, sterols (Anderson, 1932), coenzyme Q (Lester & Crane, 1959) and possibly also α-tocopherol (Green, Price & Gare, 1959), it was expected that [2-$^{14}$C]mevalonate would be exclusively incorporated into vitamin $K_2$. However, fractionation of these lipids by silicic acid column and paper chromatography revealed that the $^{14}$C-activity appeared in the neutral and esterified lipids, but not significantly in vitamin $K_2$. A comparative study on the incorporation of [2-$^{14}$C]-mevalonate and [2-$^{14}$C]acetic acid into the lipids of *M. tuberculosis* and *Mycobacterium phlei* will be described in this paper.

## MATERIALS AND METHODS

Proportions of solvents in mixtures are given by volume throughout.

*Labelled compounds.* DL-[2-$^{14}$C]Mevalonic acid lactone (15 μc/mg.) was a gift from Dr O. Isler to Professor P. S. Sarma. Samples were delactonized before use by incubating in 0·05 N-NaOH for 15 min. at 37°. Sodium [2-$^{14}$C]-acetate (60 μc/mg.) was purchased from The Radiochemical Centre.

*Cultures.* Two species of mycobacteria were used: *M. tuberculosis* $H_{37}R_v$, the human virulent strain, and *M. phlei*, the non-pathogenic strain. These were grown in the synthetic medium of Youmans & Karlson (1947) containing glycerol as the carbon source. The $^{14}$C-labelled compounds were added directly to the medium before sterilization and the bacteria were grown for the desired period (*M. tuberculosis*, 21 days and *M. phlei*, 4 days). No attempt was made to trap the respired $CO_2$. At the end of the incubation period, the media containing the bacteria were sterilized and filtered. The bacteria were washed thoroughly with distilled water and dried in a desiccator.

*Extraction and fractionation of lipids.* The dried bacteria were extracted successively with two 5 ml. portions of acetone, diethyl ether and light petroleum (boiling range 40–60°) and the pooled extract was evaporated. Vitamin $K_2$ was completely extracted by the acetone treatment. Subsequent extractions with ether and light petroleum removed the bulk of the lipids, but no more vitamin $K_2$. Further extraction of the cell residue with ethanol–ether (1:1), as used by Jacobsen & Dam (1960) for extracting vitamin K from bacteria, did not remove any more vitamin $K_2$ or lipids. However, no attempt was made to extract all the conjugated lipids. The lipids were fractionated on silicic acid–Celite columns as described by Jayaraman & Ramasarma (1961), with light petroleum, ether–light petroleum (1:9), ether–light petroleum (1:4) and ethanol successively as eluents. The ether–light petroleum (1:9) fraction, which contains vitamin $K_2$ and also some glycerides, was rechromatographed on a reverse-phase paper system (Lester & Ramasarma, 1959) and vitamin $K_2$ was detected by the modified leucomethylene blue spray (Jayaraman & Ramasarma, 1961).

*Utilization of* [2-$^{14}$C]*mevalonic acid* in vitro. The reaction mixture consisted of mevalonic acid (2·0 μmoles; 30 000 counts/min.), ATP (5 μmoles), $MgSO_4$ (5 μmoles), ascorbic acid (10 μmoles), phosphate buffer (40 μmoles; pH 7·2), menadione (1·0 μmole) and *M. tuberculosis* extract (1·6 mg. of protein) in a total volume of 0·8 ml. After incubation at 37° for 3 hr., the reaction was stopped by heating at 100° for 2 min. and the lipids were extracted by shaking twice with a mixture of 4 ml. of light petroleum and 1 ml. of ethanol. The aqueous layer was evaporated and the phosphorylated intermediates were separated by paper chromatography as described by Bloch, Chaykin, Phillips & de Waard (1959), the corresponding spots were cut out and the counts determined.

The cell-free extract was prepared by grinding the harvested cells with double the weight of glass powder, extracting with 0·25 M-tris buffer (pH 8·0) and centrifuging down the debris at 10 000*g*.

*Measurement of radioactivity.* All the samples were plated on aluminium planchets and counted in a windowless gas-flow counter and the counts were corrected for self-absorption.

Table 1. *Incorporation of* [2-$^{14}$C]*mevalonate and* [2-$^{14}$C]*acetate into* Mycobacterium tuberculosis *and* M. phlei

The cells from 75 ml. and 50 ml. of media for *M. tuberculosis* and *M. phlei* respectively were extracted. The media contained mevalonate (6·0 μmoles for *M. tuberculosis* and 3·0 μmoles for *M. phlei*) or acetate (1·0 μmole for *M. tuberculosis* and 0·5 μmole for *M. phlei*).

| | *M. tuberculosis* | | | | *M. phlei* | | | |
|---|---|---|---|---|---|---|---|---|
| | Mevalonate | | Acetate | | Mevalonate | | Acetate | |
| | (counts/min.) | (%) | (counts/min.) | (%) | (counts/min.) | (%) | (counts/min.) | (%) |
| Added to medium | 4 390 000 | 100 | 2 864 000 | 100 | 2 508 500 | 100 | 1 000 000 | 100 |
| Lost from medium | 410 000 | 9·3 | 1 549 000 | 54 | 305 000 | 12 | 845 000 | 84·5 |
| Cell residue | 290 000 | 6·6 | 704 300 | 24·5 | 5 620 | 0·22 | 42 580 | 4·3 |
| Lipids | 6 920 | 0·16 | 75 950 | 2·6 | 670 | 0·03 | 6 060 | 0·6 |
| Weight of dried cells extracted (mg.) | 258·0 | | 107·0 | | 7·5 | | 2·0 | |
| Weight of cell residue (mg.) | 245·0 | | 100·0 | | 5·2 | | 1·4 | |
| Weight of lipids (mg.) | 12·4 | | 6·0 | | 2·0 | | 0·5 | |

## RESULTS

Mevalonate was utilized by *M. tuberculosis* as shown by Table 1. The $^{14}C$-activity lost from the medium as well as that incorporated in the cell with mevalonate as substrate was lower than that with acetate. Whether this effect is because of poor utilization of mevalonate or because only one of the isomers of DL-mevalonate serves as the substrate is not clear. A greater part of the $^{14}C$-activity was left in the cell residue. This activity may represent some unconverted mevalonate or some of the phosphorylated intermediates of mevalonate metabolism (which are shown to be formed in the experiment *in vitro*, Table 6) or even some water-soluble degradation products of mevalonate within the cells. Further extraction of the cell residue with ethanol–ether (1:1) and chloroform, successively, did not remove any of this activity.

The lipids were fractionated on a silicic acid–Celite column and the distribution of the $^{14}C$-activity in the various fractions is shown in Table 2. The recoveries were poor, indicating that some highly polar components not eluted with ethanol also possessed $^{14}C$-activity. With *M. tuberculosis*, a majority of mevalonate appears to have been incorporated in the fraction eluted with ether–light petroleum (1:9) consisting of vitamin $K_2$, triglycerides and, if any, sterols and mycolic acid. On the other hand, $^{14}C$-activity from acetate was incorporated both in the ether–light petroleum (1:9) and the ethanol fractions. The acetate incorporation in the latter fraction can be expected since it consists of phospholipids among other polar compounds. The difference in the pattern of distribution of $^{14}C$-activity in ether–light petroleum (1:9) and ethanol fractions indicate that mevalonate was not incorporated via acetate. With *M. phlei* the incorporation of acetate was predominantly in the ether–light petroleum (1:9) and (1:4) fractions but with mevalonate the $^{14}C$-activity appeared in the non-polar hydrocarbon fraction representing carotenes known to be present in this organism (Goodwin & Jamikorn, 1956).

The ether–light petroleum (1:9) fraction of *M. tuberculosis* lipids was further fractionated on a second silicic acid column according to the procedure of Noll & Bloch (1955), in which mycolic acid was eluted by benzene–light petroleum (1:1) (Table 3). This fraction was evaporated, taken up in ether and extracted with 10% alkali to remove the free fatty acids. No $^{14}C$-activity was found in the free fatty acid fraction.

The fractions from chromatogram I were further separated by the reverse-phase paper chromatography in which the triglycerides remained at the origin and vitamin $K_2$ moved with $R_F$ about

Table 2. *Fractionation of the lipids on silicic acid column (chromatogram I)*

Portions of lipids applied on the columns were: samples from *M. tuberculosis*, nine-tenths for both mevalonate and acetate; from *M. phlei*, nine-tenths for mevalonate and entire amount for acetate.

| Fraction | *M. tuberculosis* | | | | | | *M. phlei* | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mevalonate | | | Acetate | | | Mevalonate | | Acetate | |
| | (counts/min.) | (%) | Lipid (mg.) | (%) | (counts/min.) | (%) | Lipid (mg.) | (%) | (counts/min.) | (%) | (counts/min.) | (%) |
| Added | 6220 | 100 | 10·8 | 100 | 68 350 | 100 | 6·0 | 100 | 600 | 100 | 6060 | 100 |
| Light petroleum | 41 | 0·7 | 0·4 | 4 | 44 | 0·07 | 0·1 | 2 | 146 | 24 | 75 | 0·1 |
| Ether–light petroleum (1:9) | 4022 | 65 | 4·6 | 43 | 16 250 | 24 | 2·5 | 42 | 47 | 8 | 1158 | 19 |
| Ether–light petroleum (1:4) | 157 | 2·5 | 0·6 | 6 | 1 550 | 2·3 | 0·5 | 8 | 54 | 9 | 599 | 10 |
| Ethanol | 603 | 9·7 | 1·8 | 17 | 12 175 | 18 | 2·5 | 42 | * | — | 151 | 2·5 |

* Less than background (about 20 counts/min.).

0·15–0·20. All ether–light petroleum (1:9) fractions showed positive spots with leucomethylene blue spray, which occasionally streaked up to $R_F$ 0·40. Table 4 shows that in all the cases the majority of the counts were found in the origin spot and only few in the region of $R_F$ 0·10–0·40 or above. From the activity in the 'vitamin $K_2$ fraction' in Table 4, taking the amount of sample into consideration, it was calculated that a total of 183 and 2260 counts/min. representing 2·6 and 3·0 % of the counts in the whole lipids were incorporated from mevalonate and acetate respectively. It was thought desirable to determine whether these counts were not because of any contamination since a larger area of paper chromatogram was eluted to include the streaked quinone spots. These

Table 3. *Rechromatography of ether–light petroleum* (1:9) *fraction of* Mycobacterium tuberculosis *lipids (chromatogram II)*

The amounts used were one-half and one-fifth of the fractions from chromatogram I for mevalonate and acetate respectively.

| Fraction | Mevalonate | | Acetate | |
|---|---|---|---|---|
| | (counts/min.) | (%) | (counts/min.) | (%) |
| Added | 2011 | 100 | 3250 | 100 |
| Light petroleum | * | — | * | — |
| Benzene–light petroleum (1:1) | 800 | 40 | 882 | 27 |
| Benzene–ether (1:1) | 485 | 24 | 2457 | 76 |
| Methanol–ether (1:9) | * | — | 215 | 6 |
| Methanol | * | — | * | — |

* Less than background (about 20 counts/min.).

Table 4. *Reverse-phase paper chromatography of fractions from chromatogram I*

Portions of fractions from chromatogram I (see Table 2) were applied to the papers. In those from *M. tuberculosis* the amounts of each fraction used were: mevalonate, ether–light petroleum (1:9), one-half; acetate, ether–light petroleum (1:9) and ethanol, one-fifth and one-tenth respectively. The entire fractions from *M. phlei* were applied.

| Fraction | *M. tuberculosis* | | | | | | *M. phlei* | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mevalonate, ether–light petroleum (1:9) | | Acetate, ether–light petroleum (1:9) | | Acetate, ethanol | | Acetate, ether–light petroleum (1:9) | | Acetate, ether–light petroleum (1:4) | |
| | (counts/min.) | (%) | (counts/min.) | (%) | (counts/min.) | (%) | (counts/min.) | (%) | (counts/min.) | (%) |
| Added | 2011 | 100 | 3250 | 100 | 1218 | 100 | 1159 | 100 | 599 | 100 |
| Origin spot ($R_F$ 0–0·1) | 1311 | 65 | 1873 | 58 | 297 | 24 | 506 | 44 | 461 | 77 |
| Middle spot ($R_F$ 0·1–0·4) | 82 | 4 | 406 | 12·5 | 50 | 4 | 86 | 7 | 34 | 6 |
| Top spot ($R_F$ 0·4–1·0) | 33 | 1·3 | 36 | 1 | 183 | 15 | * | — | * | — |

* Less than background (about 20 counts/min.).

Table 5. *Saponification of the glyceride fractions from paper chromatogram*

The origin spots ($R_F$ 0–0·1, see Table 4) were eluted and saponified.

| Fraction | Counts/min. | | Percentage of recovered counts/min. | |
|---|---|---|---|---|
| | Saponifiable | Unsaponifiable | Saponifiable | Unsaponifiable |
| *M. tuberculosis* | | | | |
| Mevalonate, ether–light petroleum (1:9) | 180 | 408 | 30 | 70 |
| Acetate, ether–light petroleum (1:9) | 481 | 493 | 49 | 51 |
| Acetate, ethanol | 133 | 91 | 59 | 41 |
| *M. phlei* | | | | |
| Acetate, ether–light petroleum (1:9) | 149 | 36 | 81 | 19 |
| Acetate, ether–light petroleum (1:4) | 93 | 90 | 51 | 49 |

Table 6. *Utilization of* [2-$^{14}$C]*mevalonate by* Mycobacterium tuberculosis *in vitro*

Details of the reaction mixture, incubation and fractionation procedures are given in the Methods section.

| Fraction | Counts/min. |
|---|---|
| Origin spot | 172 |
| $R_F$ 0·25–0·35 spot (pyrophosphomevalonate) | 129 |
| $R_F$ 0·50–0·60 spot (5-phosphomevalonate and *iso*pentenyl pyrophosphate) | 191 |
| $R_F$ 0·80–0·85 spot (mevalonate) | 26 800 |
| Lipids | 842 |

fractions were oxidized with potassium permanganate, the laevulinic acid formed was converted into its dinitrophenylhydrazone, and this was separated along with an authentic sample (Lester, Hatefi, Widmer & Crane, 1959). The hydrazones in either case showed no significant counts, showing that vitamin $K_2$ had no $^{14}$C-activity.

The glyceride fractions from the paper chromatograms (origin spots, Table 4) were eluted and were saponified with 0·2 ml. of 40% sodium hydroxide in 4 ml. of ethanol in the presence of pyrogallol for 20 min. at 100°. The $^{14}$C-activity was distributed between the saponifiable and unsaponifiable matter (Table 5). Only for the ether–light petroleum (1:9) fraction of mevalonate-incorporated lipids of *M. tuberculosis* was a greater proportion of the counts recovered in the unsaponifiable matter. The identification of the active component could not be pursued because of the small quantity of material available.

Recently Schiefer & Martius (1960) have demonstrated the synthesis of homologues of vitamin $K_2$ by isolated ox-heart mitochondria when incubated with 2-methyl-1:4-naphthaquinone and isoprene alcohol pyrophosphates. The formation of 5-phosphomevalonate, 5-pyrophosphomevalonate and *iso*pentenyl pyrophosphate from mevalonate has been demonstrated by Bloch *et al.* (1959). Since mevalonate was utilized by *M. tuberculosis*, the possibility of the formation of the phosphate intermediates and also vitamin $K_2$ was investigated with a cell-free extract. The $^{14}$C-activity was present in the phosphorylated intermediates although their identification is tentative, based only on the $R_F$ values (Table 6). The lipid extract also contained significant $^{14}$C-activity; but none of this appeared in the vitamin $K_2$ after reverse-phase paper chromatography.

## DISCUSSION

Although vitamin $K_2$ is the only well established isoprenoid compound in *M. tuberculosis*, mevalonate incorporation into it was insignificant. This is obviously not due to the inability of the cell to utilize mevalonate. The experiment with cell-free extract also showed that mevalonate can be metabolized to form the phosphorylated intermediates. It is possible that mevalonate was rapidly degraded *in vivo* to some intermediate unavailable for vitamin $K_2$ synthesis. Since acetate was also not incorporated into vitamin $K_2$, it is difficult to visualize the probable intermediate at the moment. It may be pointed out that Anderson, Norgard & Porter (1960) reported that [2-$^{14}$C]-mevalonate served as a precursor for $\beta$-carotene in *Blakeslea trispora* but not in *Chlorella pyrenoidosa*, and suggested the possibility of different mechanisms operating for isoprene synthesis in different species.

Most of the $^{14}$C-activity incorporated from mevalonate appeared in the unsaponifiable matter. The active component also appears to be more polar than hydrocarbons in elution properties. A similar incorporation of [2-$^{14}$C]mevalonate into some component in the unsaponifiable matter that is not a carotene, is not precipitated by digitonin and does not have the properties of intermediates in the squalene synthesis, has been reported in *Chlorella* by Anderson *et al.* (1960). They suggested that it could be a polyene; the presence of such a substance in *M. tuberculosis* has yet to be established. A carotene, lepretone, similar to $\beta$-carotene, has been indicated to be present in a *M. tuberculosis* strain by Takeda & Ohto (1944). Its existence in the organism used by us and also its properties are not known. If the mevalonate was not extensively degraded, other branched methyl compounds present in *M. tuberculosis*, tuberculostearic acid, mycocerosic acid and phthiocerol, could also incorporate $^{14}$C-activity from mevalonate since these can be visualized as having isoprene units with the double bond being saturated. Their biosynthesis would be of interest to study in view of their contribution to the characteristic toxicity of this bacillus.

## SUMMARY

1. [2-$^{14}$C]Mevalonic acid and acetic acid were incorporated into the lipids of *Mycobacterium tuberculosis* and *Mycobacterium phlei*. The $^{14}$C-activity was distributed in saponifiable and unsaponifiable matter.

2. It appears that mevalonic acid did not serve as a precursor of the isoprenoid side chain of vitamin $K_2$.

3. The majority of $^{14}$C-activity from mevalonic acid was found in an unidentified component of *Mycobacterium tuberculosis* lipids which appears to be more polar than are hydrocarbons in its elution properties.

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# Observations on the Effects of Methionine and Homocysteine on Growth of a Cholineless Mutant of *Neurospora crassa*

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Besides choline, *Neurospora crassa* strain no. 34486 (a cholineless mutant) can also utilize for growth monomethylethanolamine and dimethylethanolamine, the immediate precursors of choline (Horowitz, Bonner & Houlahan, 1945); this indicates that the block in synthesis of choline from ethanolamine in this mutant precedes monomethylethanolamine (Horowitz, 1946). Methionine is also active (Horowitz & Beadle, 1943) though at much higher concentrations. It was therefore of interest to determine whether homocysteine could, either partially or totally, replace methionine for growth of the mutant, and whether the addition of $C_1$ donors or pteroylglutamate, whose derivatives are recognized cofactors in transformylation reactions (Huennekens & Osborn, 1959), or both, facilitated the methylation either of monomethylethanolamine to choline or of homocysteine to methionine.

## EXPERIMENTAL

*Organism and media.* Stock cultures of the cholineless mutant no. 34486 were maintained on agar slants of the following composition (J. F. Nyc, personal communication) in g./l.: potassium tartrate, 5; $NaNO_3$, 4; $KH_2PO_4$, 1; $MgSO_4,7H_2O$, 0·5; NaCl, 0·1; $CaCl_2$, 0·1; glycerol, 20; hydrolysed casein, 0·25; yeast extract, 5; malt extract, 5; agar, 15. The pH was 5·6–5·8. The basal medium used in these experiments was the same as that used for choline assay by Horowitz & Beadle (1943).

*Growth studies.* Growth studies were carried out in 50 ml. Erlenmeyer flasks containing 10 ml. of medium. After autoclaving at 15 lb. for 15 min. the flasks were cooled and inoculated with 0·2 ml. of a spore suspension in sterile distilled water. They were incubated at 30° for 72 hr. except where stated otherwise. The mycelial pads were then removed, pressed out on filter paper, dried at 80° and weighed to the nearest 0·1 mg. Flasks were kept in duplicate and each experiment was repeated at least twice.

[Me-$^{14}$C]*Methionine uptake during synthesis.* In experiments with $^{14}$C additions, the washed mycelial pads were disintegrated in a Potter–Elvehjem glass homogenizer and made up to 6 ml. with distilled water. Portions (2 ml.) were plated on stainless-steel planchets and dried under an infrared lamp, and the radioactivity was determined directly, with the Tracerlab-SC-16 windowless gas-flow counter in conjunction with a Tracerlab-SC-51 autoscaler. Weight of dried mycelial matter on each planchet was determined to the nearest 0·1 mg.

*Determination of free sulphydryl groups.* This was by the method of Grunert & Phillips (1951).

## RESULTS

*Effects of homocysteine on growth.* Homocysteine, by itself, failed to replace choline for growth at any concentration from 5 to 400 $\mu$g./ml. Addition of DL-serine (50 $\mu$g./ml.) or formate (50 $\mu$g./ml.) as $C_1$ donors did not increase growth response over the blank, indicating a lack of methylating ability under the experimental conditions. Addition of

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pteroylglutamate (1 μg./ml.) also made no difference.

The organism could utilize methionine in place of choline to a limited extent, although maximum growth was never reached even with high concentrations. At suboptimum concentrations of DL-methionine (e.g. 20 μg./ml.) increasing concentrations of DL-homocysteine up to 75 μg./ml. markedly increased growth (Fig. 1). Further increase in homocysteine concentration, however, inhibited growth, which fell even below the basal medium blank at sufficiently high concentrations. Similar effects were noticeable at different concentrations of methionine, as well as in experiments where homocysteine solution sterilized by Seitz filtration was added aseptically to previously autoclaved basal medium.

When choline at various concentrations was substituted for methionine (Fig. 2) no stimulation of growth by homocysteine was observed and there was inhibition at higher levels of homocysteine.

The cholineless strain can utilize dimethylethanolamine and, to a lesser extent, monomethylethanolamine for growth (Horowitz *et al.* 1945). Growth either with monomethylethanolamine or with various concentrations of homocysteine in the presence of methionine was not stimulated by either serine or formate or by leucovorin.

*Nature of relationship between methionine and homocysteine.* The dual effect of homocysteine might be due to a sparing of the methionine requirement at lower concentrations and to competition between the two metabolites at higher concentrations of homocysteine. The utilization of labelled methionine was therefore followed in the presence of various concentrations of homocysteine. Radioactive methionine was added to each flask at suboptimum concentrations (20 μg./ml.) together with various concentrations of homocysteine. Activity per mg. dry weight of mycelium decreased as the concentration of homocysteine increased to 75 μg./ml. and then rose again (Table 1). On the other hand, when methionine was present at greater than optimum concentration (200 μg./ml.) the activity per mg. dry wt. did not show this trend.

*Nature of homocysteine inhibition.* The inhibitory effect of homocysteine might be attributable to its free –SH group content. However, it was observed that incubation for 18 hr. of the autoclaved basal medium containing homocysteine resulted in complete removal of the –SH groups. The –SH group was, however, unaffected in the absence of the basal medium. This disappearance could be due to (i) auto-oxidation catalysed by the metal ions in the medium, or (ii) chelation of the metal ions by the –SH groups, or both. The trace-element content of the medium (which was added as salts, in mg./l.: B 0·01, Mo 0·02, Fe 0·2, Cu 0·1, Mn 0·02 and Zn 2·0) was therefore raised. This increase of trace elements decreased both the stimulation by homocysteine up to 75 μg./ml. and the inhibition at higher concentrations when suboptimum levels of methionine were present (Fig. 3). At optimum levels of methionine, increased trace-element

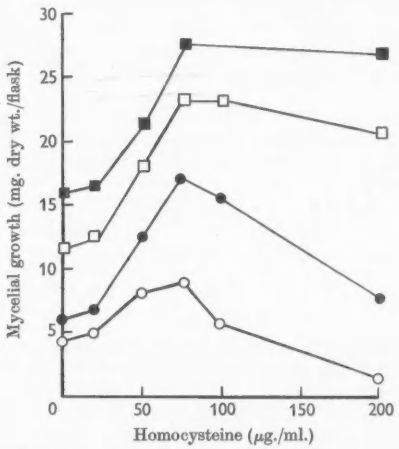


Fig. 1. Effect of various concentrations of homocysteine and methionine on growth of *N. crassa* (cholineless). The basal medium was modified with various concentrations of DL-homocysteine and DL-methionine as indicated and the extent of growth at 30° after 72 hr. was compared. Other details were as described in the text. Concn. of DL-methionine (μg./ml.): ○, 10; ●, 20; □, 40; ■, 80.

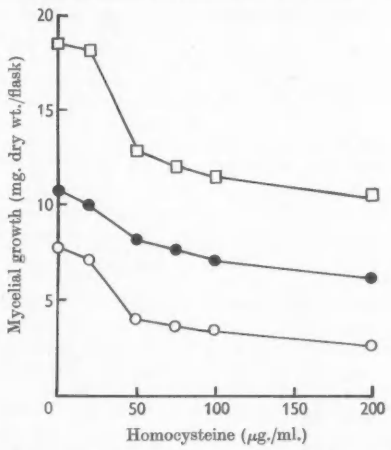


Fig. 2. Effect of various concentrations of homocysteine and choline on growth of *N. crassa* (cholineless). Details were essentially as in Fig. 1. Concn. of choline (μg./ml.): ○, 0·2; ●, 0·5; □, 1·0.

Table 1. *Incorporation of radioactive methionine into* Neurospora crassa *(cholineless) mycelium*

The results represent extent of incorporation of [Me-$^{14}$C]methionine activity into cell material of *N. crassa* (cholineless) during 72 hr. of growth on basal medium, as affected by various concentrations of homocysteine. Suboptimum methionine and excess of methionine were (per flask) 200 and 2000 $\mu$g. of DL-[Me-$^{14}$C]methionine respectively, and $9{\cdot}2 \times 10^3$ and $9{\cdot}2 \times 10^4$ counts/min. respectively.

| | Suboptimum methionine | | Excess of methionine | |
|---|---|---|---|---|
| Homocysteine ($\mu$g./flask) | Dry weight of mycelium (mg.) | Activity (counts/min./mg. dry wt.) | Dry weight of mycelium (mg.) | Activity (counts/min./mg. dry wt.) |
| Nil | 7·2 | 850 | 12·0 | 2040 |
| | 6·0 | 940 | 12·3 | 2230 |
| 200 | 7·2 | 830 | 13·2 | 2950 |
| | 7·8 | 770 | 13·2 | 2910 |
| 750 | 15·3 | 550 | 13·8 | 2430 |
| | 14·3 | 490 | 16·6 | 2220 |
| 2000 | 6·9 | 1030 | 19·5 | 2570 |
| | 6·9 | 1000 | 21·6 | 2310 |
| 4000 | 2·7 | 1000 | 18·3 | 2690 |
| | 3·0 | 940 | 18·0 | 2670 |

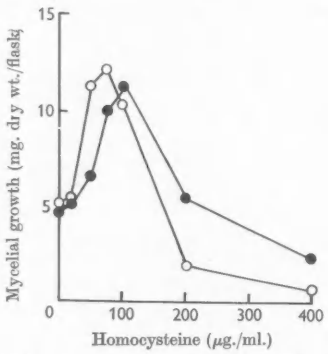


Fig. 3. Effect of concentration of trace elements on growth of *N. crassa* (cholineless). The basal medium was supplemented with 20 $\mu$g./ml. of DL-methionine. Other details were as in Fig. 1. ○, Basal trace-element concentration; ●, basal trace-element concentration increased fivefold.

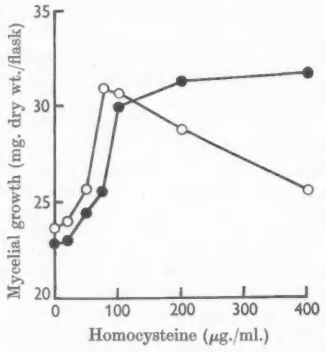


Fig. 4. Effect of concentration of trace elements on growth of *N. crassa* (cholineless). The basal medium was supplemented with 80 $\mu$g./ml. of DL-methionine. Other details were as in Fig. 1. ○, Basal trace-element concentration; ●, basal trace-element concentration increased fivefold.

concentrations in the medium completely eliminated the inhibition due to higher concentrations of homocysteine (Fig. 4).

*Studies in* Neurospora crassa *(wild)*. A similar inhibition should also be exerted on the growth of the wild strain if growth inhibition were due to chelation of metal ions by –SH groups. With the wild-type *N. crassa*, increasing the concentrations of homocysteine inhibited growth either in the presence or absence of methionine or choline (Table 2). This decrease in growth, which was nearly 50 % when homocysteine concentration was 400 $\mu$g./ml., could be restored by increasing the trace-element concentration in the basal medium (Table 3).

Table 2. *Effect of homocysteine on growth of* Neurospora crassa *(wild)*

Additions were made to the basal medium as indicated. Other details were essentially as in Fig. 1.

| Homocysteine added ($\mu$g./ml.) | Mycelial dry matter (mg./flask) with | | |
|---|---|---|---|
| | No addition | Methionine (20 $\mu$g./ml.) | Choline (0·5 $\mu$g./ml. |
| 0 | 60·0 | 64·1 | 60·4 |
| 20 | 63·7 | 63·1 | 57·6 |
| 50 | 57·1 | 60·0 | 60·5 |
| 75 | 57·2 | 57·6 | 53·0 |
| 100 | 46·2 | 53·9 | 55·8 |
| 200 | 43·6 | 43·2 | 46·9 |
| 400 | 28·3 | 26·5 | 36·7 |

Table 3. *Effect of trace elements on inhibition of growth of* Neurospora crassa *(wild) by homocysteine*

Additions of trace elements and DL-homocysteine (400 μg./ml.) were made as indicated. Other details were essentially as in Fig. 1.

| Concn. of trace elements | Mycelial dry matter (mg./flask) Without homocysteine | With homocysteine |
|---|---|---|
| Basal | 67·3 | 33·9 |
| 4-fold | 67·6 | 47·2 |
| 6-fold | 61·0 | 52·6 |
| 8-fold | 60·1 | 50·0 |
| 10-fold | 60·2 | 57·6 |
| 15-fold | 62·9 | 63·9 |
| 20-fold | 67·7 | 62·2 |

## DISCUSSION

The inability of homocysteine to support growth of the mutant strain no. 34486 of *N. crassa* in the absence of methionine indicates that it cannot totally replace methionine. This inability is not due to absence of potential $C_1$ donors since the presence of serine or formate in the basal medium has no effect.

The stimulation of growth produced by homocysteine in the presence of suboptimum concentrations of methionine (Fig. 1) could be due to a sparing effect of homocysteine for methionine. The decrease in radioactivity incorporated/mg. dry wt. of mycelium from radioactive methionine in the presence of homocysteine (Table 1), incorporation being minimal when growth stimulation is maximum, tends to confirm that homocysteine partially replaces methionine, diluting the activity incorporated into the cell material. On the other hand, the increase in growth obtained with homocysteine even in the presence of optimum concentrations of methionine (80 μg./ml.; Fig. 1) indicates that homocysteine does not exhibit a simple sparing effect which would be nullified at optimum methionine concentrations. A similar stimulation is not observed when choline replaces methionine in the medium.

The stimulatory effect of homocysteine in the presence of methionine was not accentuated by the addition of serine or formate and pteroylglutamate. Further, growth obtained with monomethylethanolamine is also not affected by similar additions, indicating that exogenous $C_1$ donors or folic acid derivatives are not utilized for methylation of monomethylethanolamine to choline, or of homocysteine to methionine. Folic acid derivatives are, however, present in the organism (Swendseid & Nyc, 1958).

Inhibition of growth at homocysteine concentrations above 75 μg./ml. (Fig. 1) could be due to competitive inhibition of methionine utilization by homocysteine, since the degree of inhibition decreases with increasing methionine concentrations. However, even at optimum concentrations of methionine, some inhibition is observable, indicating that competitive inhibition cannot be the sole cause of retardation of growth.

The considerable decrease in inhibition by homocysteine with increasing concentrations of the trace elements in the medium showed that the inhibitory effect may be due, in part at least, to chelation of the trace elements by the –SH group of homocysteine. However, the possibility cannot be precluded that the trace elements act by enhancing auto-oxidation of homocysteine to the inactive homocystine. At optimum concentration of methionine, increased concentrations of trace metals eliminated the inhibitory effect completely (Fig. 4), thus confirming the dual nature of inhibition.

The inhibition by homocysteine of growth of the wild strain, either in the presence or absence of choline or methionine (Table 2), is apparently solely due to the effect of trace elements in the medium (Table 3).

## SUMMARY

1. Homocysteine alone failed to support growth of *Neurospora crassa* mutant strain no. 34486. In the presence of suboptimum concentration of methionine, it stimulated growth up to a concentration of 75 μg./ml., beyond which inhibition was observed. These effects were independent of the presence of pteroylglutamic acid or the $C_1$ donors formate and serine.

2. Stimulation was not observed when choline was substituted for methionine.

3. Homocysteine produced inhibition of growth of wild-type *Neurospora crassa* in the presence or absence of methionine or choline.

4. Increase in concentrations of boron (as borate), molybdenum (as molybdate), $Fe^{2+}$, $Cu^{2+}$, $Mn^{2+}$ and $Zn^{2+}$ ions in the medium eliminated inhibition partially in the cholineless mutant and completely in wild-type *Neurospora crassa*.

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# Methionine Synthesis in *Ochromonas malhamensis*

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The vitamin $B_{12}$ requirement of the protozoan *Ochromonas malhamensis* is spared by methionine. The observation that glycine, in the presence of a trace of vitamin $B_{12}$, but not in its absence, could give a growth response equivalent to that given by methionine, and could also reverse inhibition produced by ethionine, has been interpreted by Johnson, Holdsworth, Potter & Kon (1957) to indicate the involvement of vitamin $B_{12}$ in methionine synthesis in this organism.

Working with resting cells and cell-free extracts of mutants of *Escherichia coli*, Woods and co-workers have demonstrated more directly that the addition of vitamin $B_{12}$ enhances the synthesis of methionine from homocysteine (Gibson & Woods, 1960; Guest, Helleiner, Cross & Woods, 1960). The involvement of folic acid derivatives in this reaction has also been demonstrated (Szulmajster & Woods, 1960; Kisliuk & Woods, 1960) with extracts of acetone-dried cells of an *E. coli* mutant requiring either serine or glycine for growth. The presence of vitamin $B_{12}$ was essential in the growth medium to confer on the extracts the ability to utilize tetrahydropteroylglutamate as a carrier of $C_1$ units derived from formaldehyde or the $\beta$-C of serine (Kisliuk & Woods, 1960).

The present work relates to certain observations on the characterization of the methionine-synthesizing enzyme in *O. malhamensis*.

## EXPERIMENTAL

*Cell growth. O. malhamensis* cells were grown in 250 ml. Erlenmeyer flasks in 100 ml. of the defined medium employed by Johnson *et al.* (1957), containing 0·4 $\mu$g. of vitamin $B_{12}$ or 0·6 g. of DL-methionine/l. as indicated. Each flask was inoculated with 0·5 ml. of a 5- to 7-day culture maintained on a medium of similar composition, fortified with either vitamin $B_{12}$ or methionine, and incubated for 5 days at 29° in a Perspex water bath illuminated by a pair of fluorescent tubes placed at a distance of 1 ft. under the bath.

The complex medium used contained (in g./l.): glucose, 10; peptone, 5; proteolysed liver extract, 2; the pH was 5·5.

The cells were harvested by centrifuging, washed twice and resuspended in ice-cold 0·2M-potassium phosphate buffer, pH 7·0, in experiments with resting cells, or in distilled water when cell-free extracts were prepared.

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*Extraction and purification of enzyme system.* Cell-free extracts were obtained by repeated alternate freezing and thawing, and subsequent centrifuging at 5000*g* for 15 min. under aseptic conditions. Microscopic examination and lack of growth response on transfer confirmed absence of viable cells.

Proteins from the extracts were fractionated by ammonium sulphate precipitation and dialysis to remove the salt. All operations were carried out at 0–5°.

*Synthesis of methionine.* Methionine synthesis was followed in the reaction system containing basically: 0·01 M-DL-homocysteine, 0·01 M-glycine, 0·1 M-potassium phosphate buffer, pH 7·0, and the enzyme preparation in a total volume of 5 ml. Incubation was at 30° under aseptic conditions. Each experiment included a zero-time control with the complete system and a mixture incubated for the experimental period without precursors.

At the end of the incubation period, the reaction was stopped by steaming for 10 min. Methionine was estimated microbiologically, in the clear supernatant by using *Lactobacillus fermenti* (Barton-Wright, 1952). DL-Methionine was used as standard and the results were expressed in terms of DL-methionine. The results in each case represent a minimum of at least four independent determinations.

*Incorporation of activity from radioactive glycine into methionine.* The incubation system was similar to the one used above except that 0·01 M-[2-$^{14}$C]glycine (activity; $1{\cdot}25 \times 10^5$ counts/min.) was used.

Methionine was isolated by a modification of the chromatographic method of Moore & Stein (1951, 1954). Inactive glycine and methionine (1 mg. each) were added as carriers to a sample of the clear supernatant which was adsorbed on Dowex 50 (200 mesh; $Na^+$ form) columns (16 mm. × 30 cm.) and eluted with 200 ml. of 0·1 M-citrate buffer, pH 3·1, followed by 200 ml. of 0·2 M-citrate buffer, pH 4·25. Methionine, free from glycine, was eluted out in the fraction between 230 and 250 ml.

Activity of the isolated methionine was determined directly at infinite thinness on glass planchets with the Tracerlab-SC-16 windowless flow-gas counter in conjunction with the Tracerlab-SC-51 autoscaler.

Methionine was determined microbiologically in a separate sample of the supernatant as described above.

*Estimation of protein.* Protein was computed from total and non-protein nitrogen values determined by microdigestion and direct nesslerization as described by Umbreit, Burris & Stauffer (1957). Protein was estimated in the ammonium sulphate precipitates with the Folin–Ciocalteu reagent (Layne, 1957).

*Determination of folic acid derivatives.* The folic acid derivatives were liberated from cell-free extracts by overnight autolysis in 0·1 M-phosphate buffer, pH 7·4, under toluene at 37° (Pfander, Dietrich, Monson, Harper &

Elvehjem, 1952; Mitbander & Sreenivasan, 1955). Folic acid activity was assayed with *Streptococcus faecalis* R turbidimetrically, by the modified medium of Mitbander & Sreenivasan (1954). *Leuconostoc citrovorum* factor activity was assayed with *L. citrovorum* (ATCC no. 8081) as the test organism, leucovorin as the standard and the basal medium of Sauberlich & Baumann (1948).

## RESULTS

### *Methionine synthesis by resting cells*

Washed cells of *Ochromonas*, grown in complex medium and incubated at 30° in 0·1 M-potassium phosphate buffer, pH 5·5, synthesized methionine from homocysteine and glycine. A rapid methionine synthesis occurred up to 10 hr., after which the rate fell off (Fig. 1). Aseptic conditions were maintained throughout harvesting and incubation procedures. The optimum pH for the reaction was 7·0 (Fig. 2) and this was used in all subsequent experiments.

*Effect of* $C_1$ *donors.* Glycine was the most effective of the $C_1$ donors tested with resting cells followed by serine, choline, methanol and betaine (Table 1). Formaldehyde and formate were ineffective. Glycine was therefore employed as a $C_1$ donor in all subsequent experiments. [2-$^{14}$C]Glycine was incorporated into methionine by the resting cells (see Table 3).

### *Experiments with cell-free extracts*

Table 2 shows the distribution of methionine-synthesizing ability in the cytoplasm and the residue of lysed cells grown in natural medium or synthetic medium containing vitamin $B_{12}$. In both cases, about 80 % of the activity was in the cell-free extract. Alternate freezing and thawing was repeated five times in these experiments.

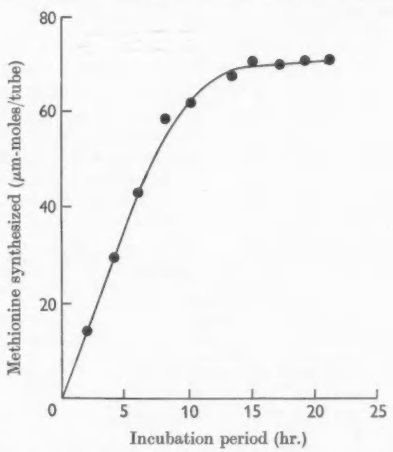


Fig. 1. Effect of period of incubation on methionine synthesis. The reaction mixture contained: DL-homocysteine, 0·01 M; glycine, 0·01 M; phosphate buffer (pH 5·5), 0·1 M; cells, 15–20 mg. dry wt.; total volume, 5 ml. The temperature of incubation was 30°.

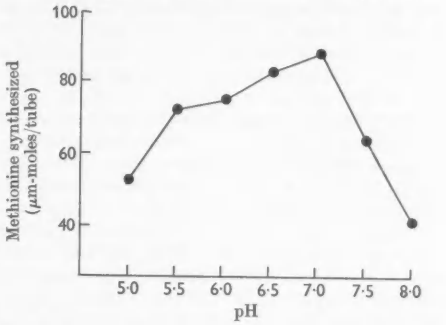


Fig. 2. Effect of pH on methionine synthesis. Details of the reaction system were as in Fig. 1. Period of incubation was 18 hr.

Table 1. *Effect of* $C_1$ *donors on methionine synthesis*

The reaction mixture contained: DL-homocysteine, 0·01 M; $C_1$ donor, 0·01 M; phosphate buffer (pH 7·0), 0·1 M; cells, 15–20 mg. dry wt.; total volume, 4 ml. Incubations were carried out for 18 hr. at 30°. In this and in all other Tables the values are given ± S.E.M.

| $C_1$ donor | Methionine synthesized (μm-moles/mg. of protein) |
|---|---|
| Nil | 10·1 ± 1·2 |
| DL-Serine | 26·1 ± 1·5 |
| Glycine | 29·2 ± 2·0 |
| Formaldehyde | 9·0 ± 0·3 |
| Methanol | 17·3 ± 1·4 |
| Choline | 19·0 ± 1·3 |
| Betaine | 15·1 ± 0·3 |
| Formate | 8·2 ± 1·1 |

Table 2. *Intracellular distribution of methionine-synthesizing ability*

The compositions of the media are described in the text. Details were essentially as in Table 1, with 0·01 M-glycine as the $C_1$ donor. Cell material used as the enzyme source was equivalent to 15–20 mg. dry wt. of cells.

| | Methionine synthesized (μm-moles/mg. of protein in cells) by organism grown in | |
|---|---|---|
| | Complex medium | Defined medium |
| Cells | 31·3 ± 1·8 | 26·6 ± 1·0 |
| Lysed cells | 25·8 ± 1·6 | 20·7 ± 1·3 |
| Cell-free extract | 19·9 ± 1·1 | 17·9 ± 2·5 |
| Cell debris | 6·1 ± 0·9 | 5·2 ± 1·2 |

*Radioactivity incorporation.* Methionine synthesis by cell-free extracts was also demonstrated by the incorporation of radioactivity into methionine on incubation with homocysteine and [2-$^{14}$C]-glycine (Table 3).

*Dowex 2 treatment.* To deplete it of cofactors, the cell-free extract containing 3–4 mg. of protein/ml. was treated with 20 % (w/v) of Dowex 2 ($Cl^-$ form; 20 mesh) in the cold for 20 min. with occasional shaking. Methionine synthesis was reduced by nearly 50 % after this treatment (Table 4). Addition of cell-free extract, previously heated to 65° for 10 min. to inactivate the enzyme system, restored activity. The activity of the untreated cell-free extract was also slightly increased by the addition of heated cell-free extract.

*Effect of cofactors.* The ability of a cofactor mixture, containing ATP, DPN, magnesium sulphate, leucovorin and vitamin $B_{12}$, to replace the heat-inactivated cell-free extract was next tested. The mixture increased methionine synthesis by both the untreated and Dowex-treated cell-free extracts, though to a lesser extent in the former case. Omission of each of the components in turn showed that the activity of this mixture was attributable to ATP (Table 4). AMP and succinate at the same concentration did not replace ATP in the above system. Sodium fluoride (0·6 $\mu$M) either by itself or in the presence of ATP had no effect, indicating a lack of adenosine-triphosphatase activity, which was subsequently confirmed by direct assay (Novikoff, Hecht, Podber & Ryan, 1952) of the cell-free extract.

Tetrahydropteroylglutamate is needed for methionine synthesis by enzyme preparations from sheep liver (Nakao & Greenberg, 1958) and from *E. coli* (Kisliuk & Woods, 1960). The activity of heated extracts in increasing methionine synthesis by the preparations from *O. malhamensis* might be due to the presence of folic acid derivatives, and attempts were therefore made to isolate these.

Dowex 2 ($Cl^-$ form) treatment reduced the activity of the cell-free extract for *S. faecalis* measured as pteroylglutamate from 6·1 to 1·6 $\mu$mg./mg. dry wt. The *Leuconostoc citrovorum* factor activity, expressed as leucovorin, was reduced from 2·0 to 0·4 $\mu$mg./mg. dry wt.

The cell-free extract (20 ml., containing 3–4 mg. of protein/ml.) was adsorbed on Dowex 2 ($Cl^-$ form; 50–100 mesh) columns (12 mm. × 15 cm.) and eluted with M-formic acid. The 15–25 ml. fraction accounted for most of the *S. faecalis* activity adsorbed on the column. Addition of this fraction, after freeze-drying and resuspension in 5 ml. of water, to the incubation system containing the Dowex-treated cell-free extract, ATP, homocysteine and glycine, did not, however, increase methionine synthesis.

Table 3. *Incorporation of activity from* [2-$^{14}$C]-*glycine into resting cells and cell-free extracts of* Ochromonas malhamensis

Resting cells, 12 mg. dry wt., and cell-free extract equivalent to 16 mg. dry wt. of cells were used per tube. [2-$^{14}$C]Glycine (0·01 M; $1·25 \times 10^5$ counts/min.) was the $C_1$ donor. Other details were as in Table 1 and text.

| | Total activity in methionine (counts/min.) | Specific activity of methionine ($\mu$c/$\mu$mole) |
|---|---|---|
| Cells | 15 950 | 1·87 |
| | 20 636 | 2·52 |
| Cell-free extract | 8 484 | 2·06 |
| | 9 815 | 2·47 |

*Ammonium sulphate fractionation of cell-free extract*

Partial purification of the methionine-synthesizing enzyme system was attempted by ammonium sulphate fractionation. The dialysed, total protein precipitate obtained by saturation of the cell-free extract with ammonium sulphate formed negligible

Table 4. *Effect of cofactor additions on methionine-synthesizing ability of cell-free extracts of* Ochromonas malhamensis

The enzyme preparation was a cell-free extract equivalent to 15–20 mg. dry wt. of cells. Other details were as in Table 2. The composition of the cofactor mixture was: ATP, 20 $\mu$moles; DPN, 2 $\mu$moles; $MgSO_4$, 10 $\mu$moles; leucovorin, 400 $\mu$g.; vitamin $B_{12}$, 0·1 $\mu$g.

| Additions | Methionine synthesized ($\mu$m-moles/mg. of protein): Untreated extract | Dowex 2 ($Cl^-$ form)-treated extract |
|---|---|---|
| Nil | 40·3 ± 2·5 | 20·9 ± 2·1 |
| Heat-inactivated cell-free extract | 48·3 ± 4·3 | 45·8 ± 3·1 |
| Cofactor mixture | 49·2 ± 1·4 | 36·2 ± 1·4 |
| Cofactor mixture without vitamin $B_{12}$ | 46·5 ± 2·2 | 34·7 ± 1·2 |
| Cofactor mixture without leucovorin | 48·1 ± 2·5 | 37·4 ± 1·9 |
| Cofactor mixture without $MgSO_4$ | 48·1 ± 2·8 | 36·3 ± 1·8 |
| Cofactor mixture without DPN | 47·6 ± 3·1 | 36·9 ± 0·8 |
| Cofactor mixture without ATP | 46·3 ± 2·1 | 23·4 ± 1·9 |

amounts of methionine ($3{\cdot}2 \pm 1{\cdot}2$ $\mu$m-moles/mg. of protein) on incubation for 6 hr. in the usual system with homocysteine and glycine as precursors. On addition of heat-inactivated cell-free extract, however, synthesis was considerably enhanced ($25{\cdot}9 \pm 2{\cdot}6$ $\mu$m-moles/mg. of protein).

On fractionation of the protein of the cell-free extract by adding ammonium sulphate, the 25–50 % fraction was found to possess maximum methionine-synthesizing activity (Table 5). Addition of heat-inactivated cell-free extract increased synthesis substantially. However, the addition of a cofactor mixture similar to that used previously failed to increase the synthesis either in the presence or absence of the heat-inactivated cell-free extract (Table 5). On dialysis the heated cell-free extract lost almost 50 % of its activity, which was restored by the cofactor mixture. The single omission of components of this mixture showed that ATP, DPN and magnesium sulphate were all necessary for maximum synthesis (Table 6).

The 50–75 % fraction could also synthesize methionine from precursors (6 $\mu$m-moles/mg. of protein in 6 hr.) and this was increased on addition of heat-inactivated cell-free extract to 43·5 $\mu$m-moles/mg. of protein. The 0–25 and 75–100 % fractions possessed negligible activity.

*Relationship with vitamin* $B_{12}$. Since vitamin $B_{12}$ has been implicated from growth studies in methionine synthesis in this organism (Johnson *et al.* 1957) methionine synthesis by cells grown in the absence of the vitamin was studied. No net synthesis of methionine could be demonstrated by the whole cells or by the cell-free extracts. Addition of vitamin $B_{12}$ (0·02 $\mu$g./ml.) had no effect. Preincubation of harvested cells for periods up to 3 hr. in fresh medium containing vitamin $B_{12}$ (0·4 $\mu$g./l.) also failed to confer methionine-synthesizing ability on the cells.

However, on addition of even trace amounts of vitamin $B_{12}$ (0·04 $\mu$g./l.) to the growth medium containing optimum concentrations of methionine for growth, both resting cells and cell-free extracts could synthesize methionine at levels comparable to that of cells grown in optimum concentrations of vitamin $B_{12}$.

These results indicated that the transformation of vitamin $B_{12}$ into an active complex was an essential prerequisite to its functioning in the methionine-synthesizing system. The effect of an extract of cells (equivalent to 5 mg. dry wt. of cells/ml. of reaction mixture) grown in the presence of the vitamin was therefore studied after inactivating the enzymes by heating at 65° for 10 min. This addition to the extracts of cells grown in a methionine-containing medium free of vitamin $B_{12}$ showed net synthesis of $12{\cdot}9 \pm 0{\cdot}7$ $\mu$m-moles of methionine/mg. of protein.

The coenzyme form of vitamin $B_{12}$ (Barker, Weissbach & Smyth, 1958; Weissbach, Toohey & Barker, 1959), dimethylbenzimidazole–vitamin $B_{12}$ coenzyme, at a concentration of 0·5 $\mu$M in the incubation system did not replace the heat-inactivated extract of cells, grown in vitamin $B_{12}$-containing medium, in the above system.

Table 5. *Effect of various additions on methionine synthesis by a partially purified enzyme preparation*

The enzyme preparation was a 25–50 % ammonium sulphate precipitate of the cell-free extract equivalent to 0·7–0·9 mg. of protein/tube. The incubation period was 6 hr. The composition of the cofactor mixture was as in Table 4. Other details were as in Table 2.

| Additions | Methionine synthesized ($\mu$m-moles/mg. of protein) |
|---|---|
| Nil | $16{\cdot}2 \pm 1{\cdot}5$ |
| Cofactor mixture | $16{\cdot}1 \pm 2{\cdot}6$ |
| Heated cell-free extract | $67{\cdot}1 \pm 2{\cdot}5$ |
| Heated cell-free extract + cofactor mixture | $62{\cdot}5 \pm 2{\cdot}4$ |
| Dialysed, heated cell-free extract | $31{\cdot}8 \pm 3{\cdot}0$ |
| Dialysed heated cell-free extract + cofactor mixture | $66{\cdot}1 \pm 3{\cdot}5$ |

Table 6. *Effect of omission of individual components of the cofactor mixture on methionine synthesis by a partially purified enzyme preparation*

Dialysed, heated cell-free extract and cofactor mixture (cf. Table 4) were added to the reaction mixture. Other details were as in Table 5.

| Omissions | Methionine synthesized ($\mu$m-moles/mg. of protein) |
|---|---|
| Nil | $66{\cdot}1 \pm 3{\cdot}5$ |
| ATP | $35{\cdot}7 \pm 3{\cdot}8$ |
| DPN | $52{\cdot}5 \pm 5{\cdot}6$ |
| $MgSO_4$ | $35{\cdot}1 \pm 2{\cdot}6$ |
| Leucovorin | $69{\cdot}9 \pm 4{\cdot}8$ |
| Vitamin $B_{12}$ | $69{\cdot}7 \pm 6{\cdot}2$ |

## DISCUSSION

The relatively high amount of methionine synthesis obtained in the presence of glycine or serine as $C_1$ donors (Table 1) indicates the need of exogenous $C_1$ sources for substantial methionine synthesis by resting cells of *O. malhamensis* and is in general agreement with results obtained in growth studies of Johnson *et al.* (1957).

Cofactor additions, of which ATP was found to be the only active component, restore activity of Dowex-2-treated cell-free extract only partially (Table 4), indicating the presence of other necessary cofactors in the cell-free extract.

The heated cell-free extract, required for optimum synthesis by the 25–50 % ammonium sulphate fraction (Table 5), was replaced by dialysed heated cell-free extract in conjunction with an

extraneous cofactor mixture whose effect was subsequently traced to ATP, DPN and $Mg^{2+}$ ions (Table 6). An unidentified, non-dialysable component, present in the cell-free extract, was apparently required for optimum synthesis since the cofactor mixture was ineffective in the absence of the dialysed extract (Table 5).

The inability of resting cells or cell-free extracts of *O. malhamensis* to synthesize methionine except in the presence of at least trace quantities of vitamin $B_{12}$ in the growth medium, indirectly demonstrated that the vitamin is required for methionine synthesis.

Resting cells or cell-free extracts of the organism grown in methionine apparently cannot convert vitamin $B_{12}$ into its active coenzyme form, unlike the cell-free extracts of the methionine–vitamin-$B_{12}$-requiring mutant of *E. coli* (Guest *et al.* 1960).

A coenzyme form of vitamin $B_{12}$, isolated from *Clostridium tetanomorphum* (Weissbach *et al.* 1959), which is active in the conversion of glutamate into $\beta$-methylaspartate in cell-free extracts of the micro-organism (Barker *et al.* 1958), propionate into succinate in *Propionibacterium* species (Stadtman, Overath, Eggerer & Lynen, 1960) and, reportedly, in amino acid incorporation into protein by microsomal pH 5 enzyme system (Mehta, Wagle & Johnson, 1959), did not, at the concentration tested, endow cell-free extracts of methionine-grown cells with the ability to synthesize methionine. Recent reports (Kisliuk, 1960; Takeyama & Buchanan, 1960) show that this factor is also not the active coenzyme form of vitamin $B_{12}$ for methionine synthesis in *E. coli*. However, the activation by heated extracts of cells grown in a vitamin-$B_{12}$-containing medium seems to indicate that the coenzyme form of vitamin $B_{12}$ in this system is not a heat-labile protein as is the vitamin $B_{12}$ coenzyme active for methionine synthesis in *E. coli* (Guest, 1959; Kisliuk, 1960). Alternatively, vitamin $B_{12}$ may be present in the heat-inactivated extract in a form that, unlike cyanocobalamin, is readily convertible into the active coenzyme form by the system.

## SUMMARY

1. Both resting cells and cell-free extracts of *Ochromonas malhamensis* grown in a vitamin-$B_{12}$-containing medium are capable of methionine synthesis. Optimum conditions of pH and reaction time, as well as the relative contribution of various precursors in the formation of the methyl moiety, have been ascertained.

2. The decrease of methionine-synthesizing activity of the cell-free extract caused by Dowex 2 ($Cl^-$ form) treatment was restored completely on addition of heat-inactivated cell-free extract and partially by adenosine triphosphate.

3. The fraction obtained by 25–50 % saturation with ammonium sulphate of the cell-free extract caused optimum synthesis of methionine in the presence of heat-inactivated cell-free extract or dialysed, heat-inactivated cell-free extract together with a cofactor mixture whose active components were adenosine triphosphate, diphosphopyridine nucleotide and magnesium sulphate.

4. Cell-free extracts of the micro-organism grown in a methionine-containing medium in the absence of vitamin $B_{12}$ only synthesized methionine in the presence of heated extracts of cells grown with the vitamin. Dimethylbenzimidazole–vitamin $B_{12}$ coenzyme did not replace the heated extract.

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# Methionine Synthesis in *Neurospora crassa*

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Horowitz and co-workers (Horowitz, 1947; Teas, Horowitz & Fling, 1948; Fling & Horowitz, 1951) have determined the pathway of methionine synthesis in *Neurospora crassa*, from genetic analysis of methionine-requiring mutant strains; it involves conversion of cysteine, via cystathionine, into homocysteine, which is methylated to methionine. A similar scheme has been suggested for methionine synthesis in *Escherichia coli* (Simmonds, 1948; Davis & Mingioli, 1950).

The involvement of vitamin $B_{12}$ in the last step, the methylation of homocysteine, has been clearly indicated in *E. coli* (Davis & Mingioli, 1950; Gibson & Woods, 1960) as well as in the vitamin-$B_{12}$-requiring protozoan *Ochromonas malhamensis* (Johnson, Holdsworth, Potter & Kon, 1957; Dalal, Rege & Sreenivasan, 1961). As vitamin $B_{12}$ is not known to be involved in the metabolism of *N. crassa*, it was considered of interest to study the biosynthesis of methionine from homocysteine in this organism.

## EXPERIMENTAL

*Cell growth.* Mycelial pads of *N. crassa* were grown in 50 ml. or 250 ml. Erlenmeyer flasks on 10 ml. or 50 ml. respectively of the medium employed for choline assay (Horowitz & Beadle, 1943), either as such, for the wild strain, or supplemented with 1 $\mu$g. of choline/ml. for the cholineless mutant strain no. 34486. The flasks were inoculated with spore suspensions of the required strain in sterile distilled water, and incubated for 60 hr. at 30°.

Grown mycelial pads were washed thrice with ice-cold distilled water and resuspended in the incubation system for experiments carried out with resting cells.

*Preparation of cell-free extracts.* The mycelial pads were disintegrated in a Potter–Elvehjem glass homogenizer in ice-cold 0·2 M-potassium phosphate buffer, pH 7·4, and then subjected to alternate freezing and thawing five times, the latter procedure accompanied by shaking with glass beads. The lysed cell suspension was centrifuged at 5000*g* for 15 min. to obtain the cell-free extract.

*Synthesis of methionine.* The incubation system employed in the study of methionine synthesis from homocysteine, by resting cells and cell-free extracts, was essentially that used for similar studies in *E. coli* (Guest, Helleiner, Cross & Woods, 1960). It contained: 0·01 M-DL-homocysteine, 0·01 M-$C_1$ donor indicated, 0·1 M-sodium succinate and the enzyme source, either resting cells or cell-free extract as indicated, in 0·1 M-potassium phosphate buffer, pH 7·4. Incubations were carried out at 37° for 4 hr.

Blanks for each experiment included a zero-hour control with the complete system, a mixture incubated for the experimental period without the precursors, homocysteine and $C_1$ donor.

At the end of the incubation period, the reaction was stopped by steaming for 10 min. and methionine estimated microbiologically in the clear supernatant with *Lactobacillus fermenti* as the test organism (Barton-Wright, 1952). DL-Methionine was used as standard and the results were also expressed in terms of DL-methionine. The presence of aminopterin at concentrations equivalent to those in the samples of reaction mixture to be assayed had no effect on the response of the organism to methionine.

The results, in each case, represent a minimum of four independent determinations.

*Determination of protein.* Protein was estimated in the cells and cell-free extracts by determining the total nitrogen, as well as non-protein nitrogen after deproteinization by 10% trichloroacetic acid, by Kjeldahl micro-digestion and direct nesslerization as described by Umbreit, Burris & Stauffer (1957) and multiplying the difference between the total and non-protein nitrogen values by 6·25.

## RESULTS

*Experiments with resting cells.* Washed mycelial pads of wild-type *N. crassa* synthesized a small amount of methionine from homocysteine on incubation for 4 hr. at 37° in 0·1 M-phosphate buffer, pH 7·4, in the presence of succinate. Addition of serine increased synthesis considerably. Table 1

Table 1. *Effect of* $C_1$ *donors on methionine synthesis by* Neurospora crassa (*wild*)

The reaction mixture contained: 0·01 M-DL-homocysteine, 0·01 M-$C_1$ donor, 0·1 M-sodium succinate, 0·1 M-potassium phosphate buffer (pH 7·4) and mycelial pad (40–50 mg. dry wt.) in a total volume of 25 ml. Incubations were carried out for 4 hr. at 37°. In this and in all other Tables the values are given ± S.E.M.

| $C_1$ donor | Methionine synthesized ($\mu$m-moles/mg. of protein) |
|---|---|
| Nil | 3·4 ± 0·8 |
| Formate | 66·9 ± 9·6 |
| Formaldehyde | 3·6 ± 0·5 |
| DL-Serine | 48·5 ± 10·8 |
| Glycine | 30·2 ± 9·7 |
| Methanol | 11·3 ± 0·6 |
| Choline | 14·2 ± 0·4 |
| Betaine | 30·1 ± 9·0 |

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gives the relative efficiency of various $C_1$ donors in influencing methionine synthesis. Formate was the most effective and was used in all subsequent experiments. Equimolar concentrations of DL-homocystine could not replace homocysteine as methionine precursor in the above system.

Resting cells of the cholineless mutant of *N. crassa* were also tested for methionine synthesis from homocysteine, by using serine, formate or glycine, the $C_1$ donors found to be most efficient with the wild type. Synthesis in all cases was less than that obtained with the wild type (Table 2).

Addition of pteroylglutamate (1 $\mu$g./ml.) to the incubation system did not affect methionine synthesis by mycelia of wild type *N. crassa* (Table 3). However, the inhibition of methionine synthesis by aminopterin demonstrates the involvement of folic acid derivatives in this reaction. Addition of leucovorin (1 $\mu$g./ml.) did not reverse this inhibition.

*Experiments with cell-free extracts.* Table 4 gives a comparison of alumina grinding and the freeze–thaw method, as procedures for preparation of cell-free extracts capable of methionine synthesis. The latter method was more effective, as there was less loss of activity during rupture, and the percentage of activity in the cell-free extract was also greater (around 80 % as compared with about 60 % with alumina grinding). The cell-free extracts used in subsequent experiments were therefore prepared by the freeze–thaw method.

Table 2. *Effect of* $C_1$ *donors on methionine synthesized by* Neurospora crassa (*cholineless*)

Details were as in Table 1.

| $C_1$ donor | Methionine synthesized ($\mu$m-moles/mg. of protein) |
|---|---|
| Nil | 3·2±0·5 |
| Formate | 19·0±7·0 |
| DL-Serine | 20·7±9·2 |
| Glycine | 13·8±3·5 |

Table 3. *Effect of folic acid derivatives on methionine synthesis*

Details were essentially as in Table 1, with 0·01 M-formate as the $C_1$ donor. Mycelial pad (25–35 mg. dry wt.) was incubated in a total volume of 10 ml.

| Additions | Methionine synthesized ($\mu$m-moles/mg. of protein) |
|---|---|
| Nil | 66·9±9·6 |
| Pteroylglutamate (10 $\mu$g.) | 62·2±6·3 |
| Leucovorin (10 $\mu$g.) | 69·6±7·0 |
| Aminopterin (10 $\mu$g.) | 43·4±2·4 |
| Aminopterin + leucovorin | 47·3±5·3 |

Table 4. *Solubilization of methionine-synthesizing system*

The procedures for rupture of cells are described in the text. Other details were as in Table 3. Cell material used as the enzyme source was equivalent to 25–35 mg. dry wt. of mycelium.

| | Methionine synthesized ($\mu$m-moles/mg. of protein in lysed cells) by cells ruptured by | |
|---|---|---|
| | Alumina grinding | Freeze–thaw |
| Lysed cells | 32·1±1·4 | 38·2±1·0 |
| Cell-free extract | 19·7±1·8 | 31·6±1·0 |
| Cell debris | 13·0±0·9 | 5·5±1·1 |

Table 5. *Restoration of activity of Dowex-2* ($Cl^-$ *form)-treated cell-free extract*

The enzyme preparation was a cell-free extract equivalent to 12–15 mg. dry wt. of cells in a total volume of 4 ml. of reaction mixture. The cofactor mixture consisted of: ATP, 20 $\mu$moles; DPN, 2 $\mu$moles; $MgSO_4$, 10 $\mu$moles; leucovorin, 400 $\mu$g. Other details were as in Table 3.

| Addition | Methionine synthesized ($\mu$m-moles/mg. of protein) | |
|---|---|---|
| | Untreated extract | Dowex-2 ($Cl^-$ form)-treated extract |
| Nil | 57·8±2·8 | 39·8±3·0 |
| Heat-inactivated cell-free extract of *N. crassa* | 54·8±3·3 | 48·2±2·5 |
| Heat-inactivated cell-free extract of *O. malhamensis* | 59·4±3·6 | 47·4±2·4 |
| Cofactor mixture | 55·7±1·8 | 46·4±1·5 |

The methionine-synthesizing capacity of the cell-free extract containing 5–6 mg. of protein/ml. was reduced by treatment with 20 % (w/v) Dowex 2 ($Cl^-$ form; 20 mesh) in the cold for 20 min. with occasional shaking. Addition of untreated cell-free extract, inactivated by heating at 65° for 10 min., partially restored the activity. Similar restoration was obtained by addition of a heat-inactivated cell-free extract of *O. malhamensis*. A cofactor mixture, containing ATP, DPN, magnesium sulphate and leucovorin, also reactivated the Dowex-treated cell-free extract to nearly the same extent (Table 5).

## DISCUSSION

The requirement of exogeneous $C_1$ sources for methionine synthesis by resting cells of *N. crassa* is demonstrated by the large increase in synthesis obtained on addition of various $C_1$ donors (Table 1). The almost equivalent effect of formate and serine in bringing about this increase indicates the

capacity of the organism to utilize $C_1$ fragments, at both the formyl and hydroxymethyl levels of oxidation, for methionine synthesis. The non-utilization of formaldehyde, which normally functions as a $C_1$ donor through the intermediate formation of hydroxymethyltetrahydropteroylglutamate (Huennekens & Osborn, 1959) is probably due to its inhibitory effect. The comparatively lower synthesis of methionine obtained with *N. crassa* (cholineless), irrespective of the $C_1$ donor used (Table 2), explains the mutant's observed requirement of methionine for growth in the absence of choline (Horowitz & Beadle, 1943).

Swendseid & Nyc (1958) have shown that *N. crassa* has a relatively high concentration of folic acid derivatives, accounting for the lack of effect of added pteroylglutamate on methionine synthesis by resting cells (Table 3). Pteroylglutamate might not be converted by the cells into the metabolically active forms of folic acid, which are probably tetrahydro derivatives of pteroylglutamate. The inhibition of growth of wild-type *N. crassa* by aminopterin (Swendseid & Nyc, 1958) is reflected in a decreased methionine synthesis in the presence of this folic acid antagonist. The inability of leucovorin to reverse the effect might be due to some unidentified types of folic acid derivatives functioning as intermediates.

The essential similarity of cofactor requirements of the methionine-synthesizing systems in *O. malhamensis* (Dalal *et al.* 1961) and *N. crassa* is demonstrated by the ability of heat-inactivated extracts of *O. malhamensis* to replace the *N. crassa* extract as a cofactor source (Table 5).

## SUMMARY

1. Resting cells of both wild-type and the mutant strain no. 34486 of *Neurospora crassa* form methionine from homocysteine. Formate is the most effective of the various $C_1$ precursors examined.

2. Added pteroylglutamate has no effect on methionine synthesis by resting cells of the wild-type organism. Leucovorin cannot restore the decrease in synthesis caused by aminopterin.

3. The methionine synthesis obtained by cell-free extracts of *Neurospora crassa* (wild) is decreased by Dowex 2 ($Cl^-$ form) treatment. Addition of heat-inactivated cell-free extracts of either *Neurospora crassa* or *Ochromonas malhamensis*, or a cofactor mixture consisting of adenosine triphosphate, diphosphopyridine nucleotide, magnesium sulphate and leucovorin, partially restores the activity.

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# Polynucleotide Phosphorylase in Ribosomes from *Escherichia coli*

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The ribonucleic acid in *Escherichia coli* has been shown to break down by two distinct routes (Wade, 1961). In the presence of ethylenediaminetetraacetic acid ribosomal ribonucleic acid breaks down into ribonucleoside 2′:3′-cyclic phosphates (*V*-route); in the presence of magnesium it breaks down into ribonucleoside 5′-phosphates (*M*-route). Although the latter process is not dependent on the presence of orthophosphate, its inclusion stimulates the process, suggesting that a polynucleotide

phosphorylase (Grunberg-Manago, Ortiz & Ochoa, 1956) may be partly responsible.

Tracer experiments with radioactive phosphorus were carried out to examine this possibility. The results are reported here.

## MATERIALS AND METHODS

*Cultural conditions. Escherichia coli* (N.C.T.C. 1100) was cultured at 37° on a Tryptone–glucose medium (TG3/W). A concentrate was prepared by mixing solutions *A*, *B* and *C* (Wade, 1961) in the proportions 7:1:2 and, when required, the medium was prepared by diluting the concentrate with 9 vol. of 55 mM-NaCl.

The cells, which were grown by continuous culture in a vessel described previously (Wade, 1961) and had a generation time of about 40 min., were washed in a solution of 0·145 M-NaCl and 5 mM-$MgCl_2$ at 2–5° and stored at −20°. They were disrupted in a Hughes (1951) press and stored at −20° for less than 4 weeks.

*Isolation of ribosomes.* A suspension of disrupted cells (7–10% wet wt. in a solution of 0·145 M-NaCl and 5 mM-$MgCl_2$) at 2–5° was mixed in an homogenizer (Townson and Mercer Ltd., Croydon, Surrey) for three periods of 10 sec., with intermittent cooling. A volume (270 ml.) was centrifuged at 25 000*g* in a Spinco model L centrifuge (no. 30 rotor) for 1 hr. at 0°. The preliminary homogenizing reduced the amount of nucleic acid deposited (Table 1) and produced a more compact pellet. The deposit was made up to 270 ml. with a solution of 0·145 M-NaCl and 5 mM-$MgCl_2$ (*R*I); the supernatant was made up to 270 ml. and centrifuged at 85 000*g* for 7·5 hr. at 0°.

The supernatant was made up to the original volume (*S*II); the deposit was made up to 90 ml. with the same salt solution and centrifuged at 85 000*g* for 7·5 hr. at 0°.

The supernatant was made up to 90 ml. (*S*III); the ribosome deposit, about 20–25% of the original disrupted preparation, was made up to 27 ml. with a solution of 0·145 M-NaCl and 2 mM-$MgCl_2$ (*R*II) and stored at 0° for less than 7 days.

In addition to the preliminary homogenizing, this fractionation differed from that previously used (Wade & Lovett, 1958; Wade, 1961) by including a third centrifuging in which the ribosomes were washed. By reducing the volume at this point advantage was taken of the greater concentration dependence of the sedimentation rate of DNA compared with that of ribonucleoprotein (Peacocke & Schachman, 1954; Tissières, Watson, Schlessinger & Hollingworth, 1959), to reduce the contamination of the latter with the former. This contamination was of the order 2% in fraction *R*II (Table 2).

*Ribosome autodegradation.* The extent of breakdown was measured by comparing the u.v. absorption (260 mμ) of an acid extract (0·1 N-$HClO_4$) at 0° with a similar extract from an alkali hydrolysate prepared by exposing a sample to 0·3 N-KOH at 37° for 18 hr.

Each acid extract was prepared in duplicate by applying 0·05 ml. to a strip of Whatman filter paper no. 542 and immersing it in 0·1 N-$HClO_4$ (Wade, 1961).

*Ion-exchange fractionation.* The acid-soluble products of RNA breakdown were extracted with 0·1 N-$HClO_4$ and fractionated by pH-gradient elution (Wade, 1960) with columns (1 cm. diam.) containing 20 ml. or 40 ml. of Dowex 1 X 2 (200–400 mesh; formate) resin. The fractions were eluted with ammonium formate under one of the following conditions: (I) 20 ml. of resin with an eluent of 0·13 M-ammonium formate flowing at 1 ml./min. and adjusted with HCl to pH 6 and to progressively lower pH values at a rate of 2·76 pH units/l. of eluent; (II) 40 ml. of resin with an eluent of 0·06 M-ammonium formate flowing at 1·7 ml./min. and adjusted with HCl to pH 5 and to progressively lower pH values at a rate of 2·44 pH units/l. of eluent.

The extinction of the eluate at 254 mμ was determined and recorded continuously (Lovett & Wright, 1961).

The radioactivity of the eluate was measured continuously by passing it through polyvinyl chloride tubing (60 cm. long, 2 mm. bore and 0·5 mm. wall) wound round a dipping counter tube type B12H (20th Century Electronics Ltd., Croydon, Surrey). This arrangement took advantage of the longer life, the ability to accept high count rates and the lower operating voltage of a halogen-quenched tube. The count rate was recorded on a chart recorder operated from a linear ratemeter type N522 (Ekco Electronics Ltd., Southend-on-Sea, Essex) and an automatic range-changing device which also protected the counter tube from excessive count rates (Lovett, 1960). The radioactivities of the bulked fractions taken from a fraction collector were measured in a liquid counter type M6H (20th Century Electronics Ltd.).

*Norit adsorption.* When further fractionation by paper ionophoresis was necessary, a nucleotide fraction (usually less than 2 μmoles) was passed through 100 mg. of Norit supported by an H3/H5 sintered-glass column (1 cm. diam.). A volume (1 ml.) of a stock suspension containing 10% of Norit SXII (Harrington Brothers Ltd., London, E.C. 1), 10% of Celite and 8% (v/v) of octan-2-ol in ethanol (Threlfall, 1957) was filtered on to the sinter and washed with 10 ml. of water. The nucleotide solution was passed through at about 2 ml./min. followed by 10 ml. of

Table 1. *Effect of homogenizing a suspension of disrupted cells upon the nucleic acid content of R*I

| Period of homogenizing (min.) | Nucleic acid sedimented in *R*I: RNA (% of total RNA) | Nucleic acid sedimented in *R*I: DNA (% of total DNA) |
|---|---|---|
| 0 | 22·7 | 19 |
| 0·25 | 19·5 | 9·7 |
| 0·5 | 16·9 | 2·43 |
| 1 | 15·9 | 2·08 |

Table 2. *Distribution of nucleic acids and nitrogen between sedimentation fractions*

The concentrations are corrected for the volume changes made during fractionation (Fig. 1).

| Sedimentation fraction | Nitrogen (mg./ml.) | RNA (mg. of purine-bound pentose/ml.) | DNA (mg./ml.) |
|---|---|---|---|
| *R*I | 0·470 | 0·135 | 0·010 |
| *R*II | 0·630 | 0·400 | 0·040 |
| *S*II | 0·510 | 0·067 | 0·235 |
| *S*III | 0·075 | 0·045 | 0·066 |

water. The nucleotide was eluted with 20 ml. of 0·05 N-$NH_3$ solution in 50% (v/v) ethanol and recovered (usually more than 90%) as a solid, by evaporating under reduced pressure from a water pump.

*Paper ionophoresis.* The reservoir-free system described by Wade & Morgan (1955) was used with the apparatus described by Lovett (1960) and a buffer of pH 3·5, composed of 3·7% (v/v) propionic acid, 0·025 N-NaOH and 0·1 mM-EDTA, or of pH 8·4, composed of 0·5 M-glycine, 0·025 N-NaOH and 0·1 mM-EDTA.

The pH 3·5 system differed from that previously used (Wade, 1961) by including a chelating agent to eliminate streaking of nucleoside polyphosphates. The chelating agent did not affect the mobilities of nucleoside monophosphates. Nucleoside 5′-mono-, 5′-di- and 5′-tri-phosphates (0·1–0·2 μmole), when applied to the paper as 4 cm. bands and subjected to 400 v for 4 hr., had the following relative mobilities: CMP, 0·14; AMP, 0·34; GMP, 0·74; UMP, 1·00; CDP, 1·00; ADP, 1·07; GDP, 1·39; UDP, 1·77; CTP, 1·62; ATP, 1·62; GTP, 1·72; UTP, 2·16. Each band was about 0·05 unit wide after ionophoresis.

The pH 8·4 system was used to separate three adenosine nucleotides that were liberated during the autodegradation of ribosomes and behaved alike during ion-exchange fractionation and during ionophoresis at pH 3·5.

Autoradiography of the ionophoresis sheets was carried out as described by Wade & Lovett (1958).

*Nucleotide analyses.* The concentrations of nucleoside 5′-monophosphates (Table 3; Fig. 2*a*) were calculated from the extinctions at 260 mμ (Wade, 1961) of bulked fractions after ion-exchange fractionation (Fig. 1). The concentrations of nucleoside 5′-diphosphates (Table 3; Figs. 2*b* and 3) produced by incubating ribosomes with inorganic [$^{32}$P]-orthophosphate, were calculated from the radioactivities of the diphosphates and the specific activity of inorganic orthophosphate. It was shown that inorganic [$^{32}$P]orthophosphate incorporated only into the terminal phosphate of these nucleotides.

The specific activities of nucleoside 5′-mono- and 5′-diphosphates (Fig. 4) were calculated from the ratio of radioactivity to u.v. absorption (260 mμ). Each nucleotide fraction from ion-exchange fractionation was concentrated by Norit adsorption, purified by paper ionophoresis at pH 3·5 and then eluted with 0·1 N-HCl.

The spectra of the nucleotide preparations were drawn by a recording spectrophotometer type CF4DE (Optica United Kingdom Ltd., Gateshead-on-Tyne, Co. Durham).

*Preparation of $^{32}$P-labelled ribonucleic acid. E. coli* (American type B) was grown in a chemically defined medium containing 2% (w/v) of glucose, 0·01 M-glutamate,

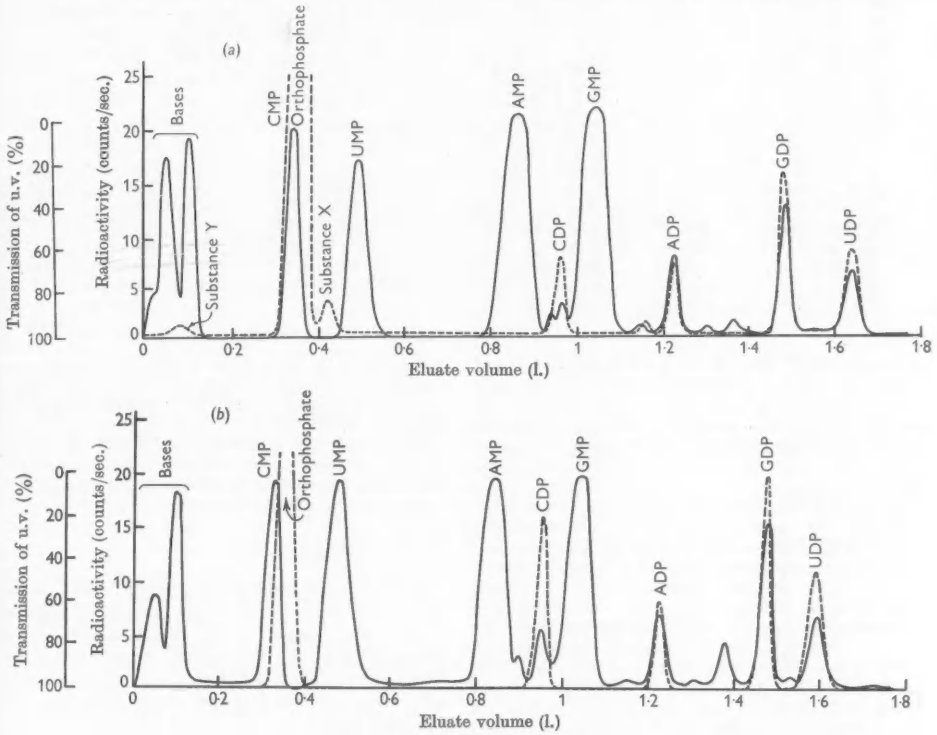


Fig. 1. For legend see following page.

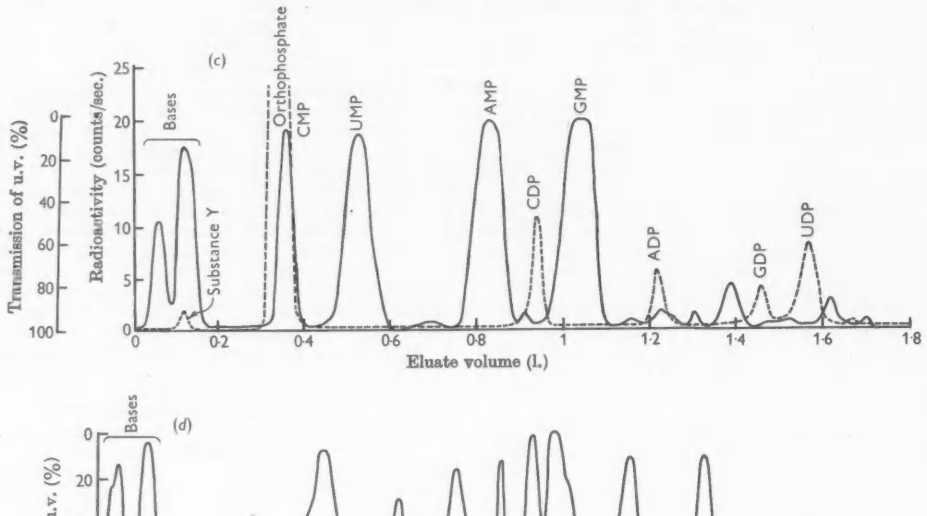


Fig. 1. Ion-exchange fractionation of the breakdown products from RNA (*a*) in disrupted cells, and (*b*, *c* and *d*) in ribosomes. (*a*) A dialysed suspension of disrupted cells (2·5 mg. of N/ml.) was incubated (37°) with mM-$MgCl_2$, 0·1 M-tris buffer, pH 8·5, and 5 mM-orthophosphate containing 10 $\mu$C of $^{32}$P/ml. at *I* 0·15 for 2 hr. Ribosomes (1·2 mg. of N/ml.) were incubated with mM-$MgCl_2$, 0·1 M-tris buffer, pH 8·5, 10 $\mu$C of inorganic [$^{32}$P]orthophosphate (*c*) with or (*b*) without 5 mM-orthophosphate at *I* 0·15 for 6 hr. (*d*) The same ribosome preparation (6 mg. of N/ml.) was stored at $-5°$ for 3 days and then incubated at a concentration of 1·2 mg. of N/ml. under the same conditions as (*c*).

0·05 M-ammonium sulphate, 4 mM-orthophosphate and 2 mc/l. of inorganic [$^{32}$P]orthophosphate in a specially designed vessel to be described in a later paper. Cells harvested during the phase of maximum exponential growth rate (45 min. doubling time) were cooled rapidly and washed with a solution of 0·145 M-NaCl and 5 mM-$MgCl_2$.

The washed cells (about 10 g. wet wt.) were disrupted in a Hughes (1951) press and the RNA was extracted with phenol (Littauer & Eisenberg, 1959). Satisfactory preparations were also carried out later with a suspension of cells (in water) which had been treated with liquid $N_2$ (Moses, 1955; Postgate, 1960). The full purification procedure of Littauer & Eisenberg (1959) was not adopted. After the RNA (about 300 mg.) had been washed with ethanol and ether, it was dried in a desiccator over $P_2O_5$ and, when required, taken up into water (10 ml.). The RNA contained 11·5% of N, 19·6% of purine-bound ribose and gave a specific extinction in water ($E^{0\cdot005\%}_{1\text{ cm.}}$) of 0·86 at 260 m$\mu$. The examination of this RNA in the analytical centrifuge (Spinco model E) revealed the presence of two main components $S_{20,w}$ 15·5 and 19·5 and one minor component $S_{20,w}$ 4·2, showing good agreement with the results of Littauer & Eisenberg (1959).

*Sources of nucleotides.* Nucleoside 5′-mono- and 5′-polyphosphates were purchased from Pabst Laboratories, Milwaukee, Wis., U.S.A. Adenosine 2′(3′):5′-diphosphate was a gift from Dr A. M. Michelson.

## RESULTS

*Breakdown of endogenous ribonucleic acid in disrupted cells in the presence of inorganic* [$^{32}$P]*orthophosphate.* Disrupted cells were suspended in an equal volume of 0·145 M-NaCl containing 5 mM-$MgCl_2$ and dialysed at 0° for 2 days against the same salt solution. The dialysed suspension was then incubated at 37° with mM-$MgCl_2$, 0·1 M-tris–HCl buffer at pH 8·5 and 5 mM-$Na_2HPO_4$ containing

20 $\mu$c of $^{32}$P/ml. The ionic strength was adjusted to 0·15 with NaCl.

After 4 hr., when about 80 % of the RNA had broken down, an acid extract was fractionated on ion-exchange resin under conditions (conditions I) which separate the 5′-mono-, 5′-di- and 5′-tri-phosphates of cytidine, uridine, adenosine and guanosine (Wade, 1960, Fig. 8). The u.v.-absorbing peaks of UMP, AMP and GMP were not associated with any radioactivity; the CMP peak was associated with the most prominent peak of activity, shown later to be entirely due to orthophosphate. The absence of radioactivity from CMP was demonstrated by passing the fraction through Norit, when the radioactivity was not retained. Of several minor u.v.-absorbing peaks, four were radioactive and were identified as CDP, ADP, GDP and UDP, from their spectra and also their behaviour during ion-exchange fractionation and paper ionophoresis at pH 3·5 and 8·4. In addition, the radioactivity of these fractions was reduced to less than 2 % when the extract was exposed to N-HCl at 100° for 10 min., conditions which hydrolyse the terminal phosphate of nucleoside 5′-diphosphates. When the individual radioactive nucleoside 5′-diphosphates were purified by paper ionophoresis and the products of acid hydrolysis subjected to ionophoresis, all the radioactivity was associated with orthophosphate, thus demonstrating that the incorporation of orthophosphate is confined to the terminal phosphate.

Under the ion-exchange conditions II, used for the analysis of nucleoside 5′-mono- and 5′-diphosphates (Tables 3 and 5; Figs. 1–4) another radioactive substance separated from the orthophosphate (Fig. 1*a*, substance X). It had no associated u.v. absorption of an intensity expected from a nucleotide.

Between 2 hr., when 70 % of the RNA had broken down, and 4 hr. the concentrations of bases and nucleoside 5′-phosphates increased slightly and the concentrations of nucleoside 5′-diphosphates diminished (Table 3); there was no significant change in the concentrations of substance X.

The inclusion of 5 mM-arsenate in the mixture did not affect the rate of breakdown or the extent of breakdown after 2 hr. but the concentrations of bases were slightly lower and the concentrations of nucleoside 5′-phosphates slightly higher than in its absence. The concentrations of CDP, UDP and ADP were markedly reduced; the concentration of GDP was only slightly reduced (Table 3).

*Conditions which influence the rate of the* M*-route autodegradation of ribosomes.* The breakdown of ribosomal RNA, like the breakdown of RNA in an unfractionated suspension of disrupted cells (Wade, 1961), was stimulated by orthophosphate (Table 4).

Table 3. *Products of* M*-route breakdown with inorganic* [$^{32}$P]*phosphate*

A dialysed suspension of disrupted cells (2·5 mg. of N/ml.) was incubated (37°) with mM-$MgCl_2$, 0·1 M-tris buffer, pH 8·5, and 5 mM-orthophosphate containing 20 $\mu$c of $^{32}$P/ml. at *I* 0·15. A ribosome preparation (1·38 mg. of N/ml.) was incubated with mM-$MgCl_2$, 0·1 M-tris buffer, pH 8·5, and 10 mM-orthophosphate containing 20 $\mu$c of $^{32}$P/ml. at *I* 0·15.

| | Period of incubation (hr.) | Rate of breakdown (%/hr.) | Bases and nucleoside monophosphates | | | | | Extent of orthophosphate incorporation into substance X and nucleoside 5′-diphosphates | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Bases (mM) | CMP (mM) | UMP (mM) | AMP (mM) | GMP (mM) | X (mM) | CDP (mM) | UDP (mM) | ADP (mM) | GDP (mM) |
| Disrupted cells | 2 | 35·5 | 2·25 | 1·87 | 1·29 | 1·66 | 2·05 | 0·076 | 0·047 | 0·131 | 0·041 | 0·095 |
| | 4 | — | 2·77 | 2·03 | 1·22 | 1·87 | 1·94 | 0·072 | 0·019 | 0·026 | 0·030 | 0·043 |
| Disrupted cells with arsenate (5 mM) | 2 | 37 | 1·58 | 1·82 | 1·66 | 1·79 | 2·20 | — | 0·018 | 0·057 | 0·018 | 0·074 |
| Ribosomes | 2 | 17·6 | 0·18 | 0·61 | 0·72 | 0·76 | 1·14 | 0 | 0·148 | 0·112 | 0·086 | 0·221 |
| | 6 | — | 1·40 | 1·32 | 1·57 | 1·52 | 2·33 | 0 | 0·220 | 0·124 | 0·115 | 0·275 |

Table 4. *Effect of orthophosphate and freezing upon the autodegradation of ribosomes*

Two suspensions (*a* and *b*) of ribosomes (1·2 mg. of N/ml.), undialysed or dialysed at 0° for 2 days against a solution of 0·145 M-NaCl and 5 mM-$MgCl_2$, were incubated at 37° with mM-$MgCl_2$, 0·1 M-tris buffer, pH 8·5, *I* 0·15. The rate was measured over the first 2 hr.

| Concn. of ortho-phosphate (mM) | Rate of breakdown of RNA (%/hr.) | | |
|---|---|---|---|
| | Undialysed preparations | | Dialysed preparation |
| | (*a*) | (*b*) | (*a*) |
| 0 | 16·5 | 12·5 | 8·5 |
| 1 | 17 | 12·5 | 8·5 |
| 2 | 18·5 | 14·3 | 8·5 |
| 5 | 21 | 16·7 | 10·5 |
| 5 | — | 37* | — |
| 10 | 26·5 | 21·0 | 14·5 |

* The rate of breakdown after the ribosome preparation had been stored at −5° for 3 days before incubation.

The variation in the rate of the process observed from one preparation to another is indicated by the results of two experiments (*a* and *b*) in Table 4. The rate of breakdown with mM-$MgCl_2$ and 5 mM-orthophosphate usually falls between 15 and 22 %/hr.

The rate was not greatly influenced by the concentration of magnesium (0·4–2 mM) in the presence of 5–10 mM-orthophosphate, by the period of storage (1–11 days) of the preparation at 0° or by the concentration of ribosomes (0·3–1·2 mg. of N/ml.) during incubation at 37°. There was, however, a tendency for the extent of breakdown to increase from 85 to 95 % as the concentration of ribosomes during incubation was reduced.

Dialysis against a solution of 0·145 M-NaCl and 5 mM-$MgCl_2$ reduced the rate to about one-half over the first hour's incubation with mM-$MgCl_2$ and 5 mM-orthophosphate (Table 4).

Arsenate (5 mM) did not affect the rate of the process in the presence of mM-$MgCl_2$ and 5 mM-orthophosphate. The rate was substantially increased when the ribosome preparation was frozen at −5° for 3 days before being incubated (Table 4). This treatment also caused a marked change in the acid-soluble products (Fig. 1*d*) in a way to be described below.

*Autodegradation of ribosomes in the presence of inorganic* [$^{32}$P]*orthophosphate.* A similar experiment to the one described above with an unfractionated preparation of disrupted cells, was carried out with ribosomes. The latter were incubated with mM-$MgCl_2$ and 10 mM-orthophosphate containing 20 $\mu$c of $^{32}$P/ml. After incubating for 2 hr. about 45 % of the RNA had broken down; after 6 hr. about 90 % had broken down. As before, the radioactivity in the products was associated with nucleoside 5′-diphosphates; the extent of incorporation into nucleoside 5′-diphosphates, however, did not diminish towards the end of the process (Table 3).

The specific activities of CDP, UDP, ADP and GDP, produced by incubating ribosomes (1·2 mg. of N/ml.) with mM-$MgCl_2$ and 5 mM-orthophosphate containing 10 $\mu$c of $^{32}$P/ml. for 6 hr., were 380, 367, 352 and 322 counts/min./$\mu$m-mole respectively. The close agreement with the specific activity of the orthophosphate present (360 counts/min./$\mu$m-mole) suggested the absence of any significant incorporation of other phosphates into this site and enabled the concentration of each nucleoside 5′-diphosphate to be calculated from its radioactivity and the specific activity of orthophosphate.

During incubation of ribosomes with mM-$MgCl_2$ and 5 mM-orthophosphate, nucleoside monophosphates were formed at a constant rate until about 80 % of the RNA had broken down (Fig. 2*a*); the rate of formation of diphosphate, however, fell off when about 40 % of the RNA had broken down (Fig. 2*b*). The influence of orthophosphate concentration upon the prominence of nucleoside 5′-diphosphates in the products was also examined.

Ribosomes were incubated with mM-$MgCl_2$ and inorganic [$^{32}$P]orthophosphate without added unlabelled orthophosphate and with concentrations of up to 20 mM-orthophosphate. The period of incubation was adjusted (3–9 hr.) to ensure the breakdown of between 80 and 90 % of the RNA. The concentration of each base fraction (Fig. 1) or nucleoside 5′-phosphate was determined from the u.v. absorption of the ion-exchange fraction. In addition to the nucleoside 5′-diphosphates, a radioactive substance Y (Fig. 1*c*) was produced, which was very prominent in the presence of low concentrations of orthophosphate.

Some incorporation of $^{32}$P occurred into nucleoside 5′-diphosphates and substance Y in the absence of added unlabelled orthophosphate (Fig. 1*c*). After incubation for 9 hr., about 0·3–0·6 mM-orthophosphate is normally liberated from the RNA; however, initially, the concentration of orthophosphate is less than 0·1 mM.

As the concentration of unlabelled orthophosphate was increased to 5 mM, the concentrations of CDP, UDP and ADP increased proportionately (Fig. 3). The concentration of substance Y, on the other hand, remained constant at about 1·5 $\mu$M (with respect to the incorporated orthophosphate). The concentration of GDP did not change proportionately with the other nucleoside 5′-diphosphates: in the presence of mM-orthophosphate it was the least prominent diphosphate, in the presence of more than 2 mM-orthophosphate it was the most prominent.

The results in Fig. 2 suggested that the formation

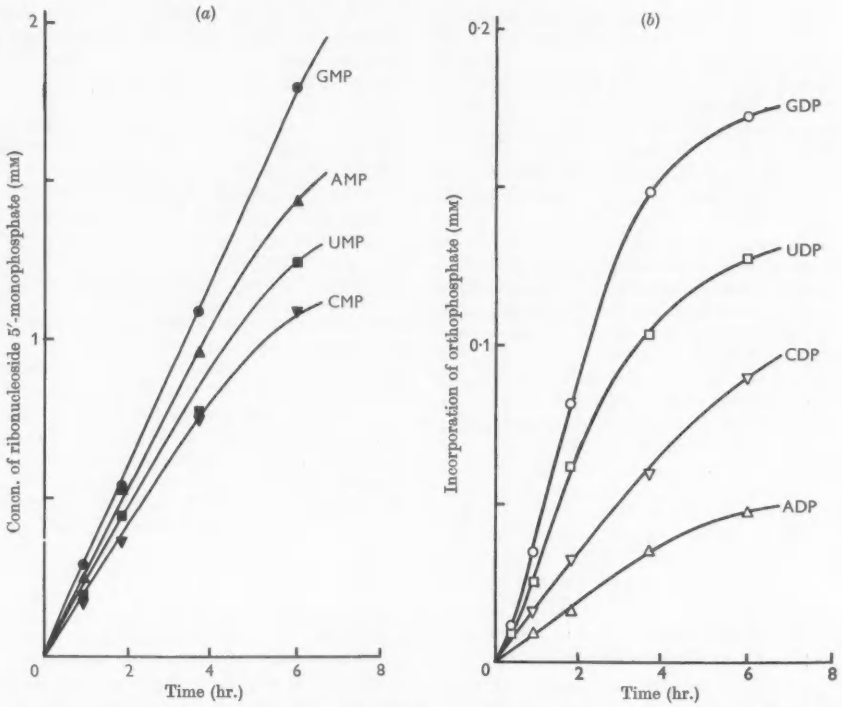


Fig. 2. Formation of (*a*) nucleoside 5′-monophosphates and (*b*) nucleoside 5′-diphosphates during the autodegradation of ribosomes with orthophosphate. Ribosomes (1·2 mg. of N/ml.) were incubated (37°) with mM-$MgCl_2$, 0·1 M-tris buffer, pH 8·5, and 5 mM-orthophosphate containing 10 $\mu$C of $^{32}$P/ml. at *I* 0·15.

of nucleoside 5′-monophosphates was independent of the formation of nucleoside 5′-diphosphates. With 20 mM-orthophosphate, the competition between the two systems for the available RNA became more evenly balanced (Table 5).

Dialysis of the ribosomes against 0·145 M-NaCl and 5 mM-$MgCl_2$ had no significant influence upon the prominence of nucleoside 5′-diphosphates produced during incubation with 1–2 mM-$MgCl_2$ in the presence or the absence of 5 mM-orthophosphate.

Arsenate (5 mM) reduced the prominence of nucleoside 5′-diphosphates without altering the rate of the process. When the ribosome preparation was allowed to freeze (−5°) for 3 days before being incubated with mM-$MgCl_2$ and 5 mM-orthophosphate the rate was greatly increased (Table 4) and a more complicated process of degradation took place (Fig. 1*d*). Nucleoside 5′-diphosphates were produced but in much smaller quantities.

The relationship between orthophosphate concentration, the rate of breakdown and the prominence of nucleoside 5′-diphosphates in the products revealed by these experiments could be explained in two ways. The diphosphates could be produced by the phosphorylation of the monophosphates by using energy derived from RNA degradation or directly by the phosphorolysis of the RNA. Evidence of the absence of any equilibrium between the monophosphates and diphosphates of each nucleoside (Table 3, Fig. 2) suggested that the formations of nucleoside 5′-monophosphates and nucleoside 5′-diphosphates were independent, favouring the latter hypothesis. More conclusive evidence was sought by following the fate of $^{32}$P-labelled RNA added to a suspension of ribosomes which had been exhaustively degraded by the *M*-route.

*Breakdown of exogenous* $^{32}$P*-labelled ribonucleic acid.* A suspension of ribosomes (1·2 mg. of N/ml.) was incubated for 6 hr. at 37° when the breakdown of endogenous RNA was complete. Sodium ribonucleate, labelled with $^{32}$P, was then added and the changes in specific activity of the nucleoside 5′-mono- and 5′-di-phosphates formed were followed

over a period of 45 min. When this experiment was first carried out, extensive degradation of the sodium ribonucleate into other nucleotides occurred, probably through the chelation of $Mg^{2+}$ ions by the ribonucleate resulting in the stimulation of the *V*-route of breakdown (Wade, 1961). When a concentration of $MgCl_2$ equivalent to the molar concentration of RNA-nucleotide was included, the breakdown was more closely confined to the *M*-route.

After a preliminary fractionation on ion-exchange resin of each acid extract under conditions II, the nucleotides were concentrated on Norit and further purified by paper ionophoresis at pH 3·5 before the specific activities were determined. As previous experiments had shown that the orthophosphate is incorporated specifically into the terminal phosphate of nucleoside 5′-diphosphates, the specific activities derived (Fig. 4) were those of the phosphate group adjacent to the ribose, the terminal phosphate being unlabelled.

The low specific activity of the orthophosphate during this period (Fig. 4) demonstrated the low phosphatase activity of the ribosome preparation and also suggested that, had any substantial exchange between the orthophosphate and the

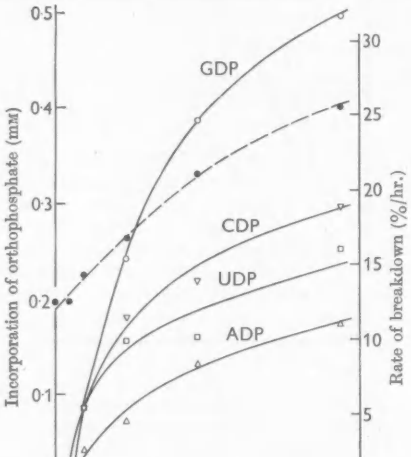


Fig. 3. Influence of orthophosphate concentration upon the concentrations of nucleoside 5′-diphosphates in the products of ribosome autodegradation. Ribosomes (1·2 mg. of N/ml.) were incubated (37°) with mM-$MgCl_2$, 0·1 M-tris buffer, pH 8·5, with 10 $\mu$c of inorganic [$^{32}$P]orthophosphate and different concentrations of unlabelled orthophosphate at *I* 0·15 for 9 hr. (mM-orthophosphate), 6 hr. (2 mM- and 5 mM-orthophosphate), 4 hr. (10 mM-orthophosphate) or 3 hr. (20 mM-orthophosphate). ●, Rate of RNA breakdown (%/hr.).

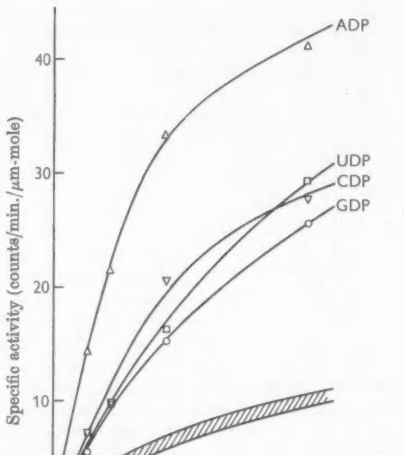


Fig. 4. Products of $^{32}$P-labelled RNA, degraded by an unlabelled ribosome preparation. Ribosomes (1·2 mg. of N/ml.) were incubated (37°) with mM-$MgCl_2$, 0·1 M-tris buffer, pH 8·5, and 10 mM-orthophosphate at *I* 0·15 for 6 hr. $MgCl_2$ (9 mM) and $^{32}$P-labelled RNA (about 1 mg./ml.) were then added and the products liberated during the following 45 min. examined. ●, Orthophosphate; ▨, nucleoside 5′-phosphates.

Table 5. *Influence of orthophosphate upon the rate of formation of nucleoside 5′-monophosphates and nucleoside 5′-diphosphates*

Ribosomes (1·3 mg. of N/ml.) were incubated (37°) with mM-$MgCl_2$, 0·1 M-tris buffer, pH 8·5, *I* 0·15, with 20 $\mu$c of $^{32}$P/ml. The rates of formation of nucleotides were measured during the breakdown of 30% of the total RNA.

| Orthophosphate concn. (mM) | Rate of formation of nucleotides during the autodegradation (mM/hr.) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | CMP | UMP | AMP | GMP | CDP | UDP | ADP | GDP |
| 5 | 0·18 | 0·22 | 0·27 | 0·27 | 0·016 | 0·029 | 0·009 | 0·04 |
| 20 | 0·35 | 0·32 | 0·52 | 0·52 | 0·22 | 0·26 | 0·12 | 0·36 |

terminal phosphate occurred, it would not have affected the specific activities of the nucleoside 5′-diphosphates.

The marked differences between the mono- and di-phosphates (Fig. 4) in fact suggested the absence of any rapid equilibrium between the two and the higher specific activities of the diphosphates strongly suggested that these could not have arisen from the monophosphates by phosphorylation but were more likely derived from the RNA by phosphorolysis.

The exceptionally high specific activity of ADP (Fig. 4) was shown later to be due to its contamination with two other adenosine nucleotides during ion-exchange fractionation and ionophoresis at pH 3·5. The three nucleotides were separated by ionophoresis at pH 8·4, when the contaminating nucleotides had mobilities of 0·78 and 1·25 with respect to ADP. The fastest nucleotide behaved like adenosine 2′(3′):5′-diphosphate during ion-exchange fractionation and during paper ionophoresis at pH 3·5 and 8·4. After the autodegradation of ribosomes with inorganic [$^{32}$P]orthophosphate only ADP, of the three, became radioactive.

*Insignificance of contamination from RI*

The above experiments demonstrated the presence of two, apparently independent, processes of ribosome autodegradation in the presence of magnesium: one occurs in the absence of orthophosphate and produces nucleoside 5′-phosphates (Fig. 1*b*); the other occurs together with the first in the presence of orthophosphate and produces nucleoside 5′-diphosphates (Fig. 1*c*). The question arose whether or not the enzymes responsible are present in the ribosomes or are contaminants from other sedimentation fractions. Since the ribosomes first sedimented are washed by a second centrifuging the most likely contamination was from fraction *RI*. This was all the more plausible since occasionally a small quantity of turbid material drains from the deposit *RI* when the supernatant is poured off, and polynucleotide-phosphorylase activity has already been reported in particles from *Ascaris lumbicoides*, which sediment like *RI* (Entner & Gonzalez, 1959). A previous experiment (Wade, 1961, Fig. 4) had shown that the removal of *RI* from a suspension of disintegrated cells does not influence the rate at which exogenous RNA is broken down in the presence of 0·15 M-orthophosphate, suggesting that a polynucleotide phosphorylase is not present in this fraction. The activity of *RI* towards the breakdown of ribosomal RNA, however, has not been examined. A sedimentation fractionation was therefore carried out in which the period of the first centrifuging was increased to 3·5 hr., resulting in about one-quarter of the ribosomes sedimenting in fraction *RI*. The fraction *RII* produced in this way was then subjected to autodegradation with mM-$MgCl_2$ both in the presence and in the absence of 5 mM-orthophosphate.

The results were similar to those illustrated in Figs. 1*b* and *c*, suggesting that the enzymes responsible for the formation of nucleoside 5′-monophosphates and nucleoside 5′-diphosphates are intimately associated with, or a part of, the ribosomes.

## DISCUSSION

Earlier experiments (Wade, 1961) showed that ribosomes from *E. coli* are capable of breaking the RNA moiety into ribonucleoside 2′:3′-cyclic phosphates in the presence of a chelating agent (*V*-route) and into nucleoside 5′-monophosphates in the presence of $Mg^{2+}$ ions (*M*-route). At that time, the rate of breakdown into nucleoside 5′-monophosphates was observed to be stimulated by orthophosphate but the formation of nucleoside 5′-diphosphates was obscured by the presence of other nucleotides.

The specific labelling of nucleoside 5′-diphosphate during the *M*-route process in the presence of inorganic [$^{32}$P]orthophosphate suggests that one of the three following schemes operated:

(1) RNA + orthophosphate → nucleoside 5′-diphosphates → nucleoside 5′-monophosphates + orthophosphate

(2) RNA → nucleoside 5′-monophosphates. Nucleoside 5′-monophosphates + orthophosphate → nucleoside 5′-diphosphates

(3) RNA → nucleoside 5′-monophosphates. RNA + orthophosphate → nucleoside 5′-diphosphates.

The fact that breakdown into nucleoside 5′-monophosphates occurs at an appreciable rate even in the absence of orthophosphate (Table 4, Fig. 3) strongly suggests that the formation of monophosphates is independent of the formation of diphosphates. It is unlikely therefore that scheme (1) is correct since the formation of monophosphates would be dependent upon the presence of orthophosphate. It may be argued that only traces of orthophosphate would be required; in this event it is surprising that changes in the concentration of orthophosphate at much higher levels (5–20 mM) should influence the rate of the process.

Scheme (2) is not supported by the experiment with $^{32}$P-labelled RNA in which the diphosphates had higher specific activities than the monophosphates at all times (Fig. 4).

The interpretation which best fits the results is summarized in (3). The *M*-route is largely the result of production of nucleoside 5′-phosphates by a phosphodiesterase. In the presence of ortho-

phosphate, however, a polynucleotide phosphorylase contributes to the degradation. One interesting similarity between the latter phosphorylase and the one described by Grunberg-Manago *et al.* (1956) is the marked influence the concentration of orthophosphate has upon the activity of the enzyme towards GDP (Singer, Hilmoe & Grunberg-Manago, 1960). As the concentration of orthophosphate is increased, the prominence of GDP in the product increases relative to the other diphosphates (Fig. 3).

The constant rate of formation of nucleoside 5′-monophosphates during autodegradation in the presence of orthophosphate (Fig. 2*a*) is consistent with the reactions being confined to the terminal residues and, in this respect, it behaves like the phosphodiesterase from snake venom (Razzell & Khorana, 1959). The earlier deterioration in the rate of formation of nucleoside 5′-diphosphates (Fig. 2*b*) can be attributed to the equilibrium constant of the phosphorylase being less completely towards the formation of nucleotides (Grunberg-Manago *et al.* 1956).

The identity of substance X (Fig. 1*a*) has not been pursued. Its absence from the products of ribosomes (Fig. 1*b* and *c*) suggests that it is produced by an enzyme that is not present in this sedimentation fraction of the cell.

For reasons stated previously (Wade, 1961) the function of polynucleotide phosphorylase in the ribosome fraction is not clear. One possibility, however, is that its substrates *in vivo* are oligonucleotides with terminal pyrophosphate groups.

## SUMMARY

1. The stimulation of ribosome autodegradation into ribonucleoside 5′-phosphates by orthophosphate, has been examined by using inorganic [$^{32}$P]phosphate and $^{32}$P-labelled ribonucleic acid.

2. Dialysed preparations of disrupted *Escherichia coli* incorporated inorganic [$^{32}$P]orthophosphate into the terminal phosphate of ribonucleoside 5′-diphosphates and also into an unidentified substance X.

3. Arsenate did not alter the rate of degradation of ribonucleic acid but reduced the extent of incorporation into nucleoside 5′-diphosphates and inhibited the formation of substance X.

4. A ribosome preparation, incubated under similar conditions, also produced ribonucleoside 5′-diphosphates with $^{32}$P confined to the terminal phosphates of nucleoside 5′-diphosphates but not substance X. Dialysis reduced the rate of the process by about 50 %. Arsenate did not affect the rate but reduced the concentrations of nucleoside 5′-diphosphates produced.

5. An increase in the rate of the process, as the concentration of orthophosphate was increased, was accompanied by a progressive increase in the concentrations of nucleoside 5′-diphosphates in the products. The relative concentrations of cytidine 5′-diphosphate, adenosine 5′-diphosphate and uridine 5′-diphosphate produced in the presence of mM–20 mM-orthophosphate remained about constant; guanosine 5′-diphosphate, however, changed from the least prominent to the most prominent diphosphates as the concentration of orthophosphate was increased from mM to 2 mM.

6. After the exhaustive autodegradation of ribosomes and the addition of $^{32}$P-labelled ribonucleic acid, the specific activities of the nucleoside 5′-diphosphates exceeded those of the nucleoside 5′-monophosphates, suggesting that the nucleoside 5′-diphosphates are produced directly from ribonucleic acid by phosphorolysis.

7. The results suggest that both a polynucleotide phosphodiesterase and a polynucleotide phosphorylase are present in ribosomes from *Escherichia coli* and that the phosphorylase, during the early stages of the process, accounts for 14 or 36 % of the total activity when 5 mM- or 20 mM-orthophosphate respectively is present.

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# The Interaction of 'Soluble' Ribonucleic Acid, Magnesium Ions and Sulphydryl Groups in the Control of Amino Acid-Dependent Pyrophosphate-Exchange Reactions

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During an attempt to purify a leucine-activating enzyme from pig-liver-protein fractions low in 'soluble' ribonucleic acid (Hele & Finch, 1960), some fractions became very unstable and lost over half their activity when kept overnight at $-15°$. In some instances, enzyme activity has been found to be preserved by the presence of 'soluble' ribonucleic acid. In this paper the effect has been further studied with preparations from rat liver. The evidence suggests that the terminal nucleotide sequence of the 'soluble' ribonucleic acid (Hecht, Stephenson & Zamecnik, 1959) is particularly involved in conferring protection upon the enzyme system. Also, removal of 'soluble' ribonucleic acid from preparations of the leucine-activating enzyme greatly enhances the pyrophosphate exchange at high concentrations of $Mg^{2+}$ ion, and the terminal nucleotide sequence (Hecht *et al.* 1959) again seems to be particularly concerned.

The work has also been extended to the activation of isoleucine and lysine. Several investigators (Rendi & Hultin, 1959; Hoagland, Keller & Zamecnik, 1956; Novelli, 1958) have found little or no ability to activate lysine, as determined by the pyrophosphate-exchange reaction, in extracts from rat liver. Rat-liver-protein fractions low in 'soluble' ribonucleic acid (Hele & Finch, 1960) have now been found to catalyse a vigorous lysine-dependent pyrophosphate exchange in the presence of unexpectedly high concentrations (above 20 mM) of $Mg^{2+}$ ions; at 5 mM concentration of $Mg^{2+}$ ions this enzyme cannot be detected.

I have also observed some effects of oxidized and reduced glutathione and of *p*-chloromercuribenzoate on the activity and stability of the leucine- and lysine-activating enzymes.

Preliminary accounts of this work have already been published (Hele, 1960*a*, *b*).

## MATERIALS AND METHODS

*Chemicals.* ATP (disodium salt), AMP, tris, L-leucine, L-isoleucine and L-valine were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Tris was titrated to the required pH with HCl. ATP was neutralized to pH 7·2 with NaOH or $MgCO_3$ (indicator paper) and AMP likewise neutralized with NaOH or tris. L-Lysine and L-phenylalanine were supplied by the California Corp. for Biochemical Research, Los Angeles 63, Calif., U.S.A. DL-Methionine, DL-threonine, L-tryptophan and protamine sulphate (salmon) were obtained from British Drug Houses Ltd. The preparation of protamine sulphate solutions has already been described (Hele & Finch, 1960). Another sample of protamine sulphate (salmon) and *p*-chloromercuribenzoic acid (sodium salt) were obtained from L. Light and Co. Ltd. *p*-Chloromercuribenzoic acid was prepared as a 2·5 mM solution in 20 mM-tris–HCl, pH 8·4, by suspension in the buffer and treating with ultrasonic vibrations for 30–60 sec. (H. R. Levy, personal communication). Glutathione, predominantly in the reduced form (GSH), was obtained from The Distillers (Biochemicals) Co. Ltd., Liverpool, and was neutralized to pH 7·2 with NaOH or tris (indicator paper). Oxidized glutathione (GSSG) was prepared by passing a stream of oxygen through a 100 mM solution of GSH, pH 7·2, until only about 2% of the nitroprusside-reacting material remained (Grunert & Phillips, 1951). Crystalline ribonuclease was obtained from C. F. Boehringer und Soehne, Mannheim, Germany. EDTA was neutralized to pH 7·4 with KOH. The source and treatment of the yeast ribonucleic acid and the charcoal have been described by Hele & Finch (1960). Ammonium sulphate was either recrystallized from water or, when indicated, from mM-EDTA. One batch of each was prepared, and the same batches were used throughout all the experiments described here. Solutions of $(NH_4)_2SO_4$, saturated at 4°, were neutralized with tris to pH 6·5. The tris was added to the saturated solution in the cold, but the pH was determined at room temperature with a 1 in 5 dilution of the saturated solution. The mixture of recrystallized $(NH_4)_2SO_4$ and potassium acetate, used as an enzyme inhibitor, contained 530 mM-$(NH_4)_2SO_4$ and 100 mM-potassium acetate; the pH was about 7·4. Sucrose, freed from traces of heavy metals by chromatography on Amberlite resin (monobed MB-1), and used in a few experiments, was a generous gift from Mr D. Tyler of this Unit. Calcium triphosphate gel was prepared according to Keilin & Hartree (1938). Stock solutions of sodium phosphate, pH 8·0, from which the 'elution mixture' was prepared, were obtained by titrating M-$Na_2HPO_4$ with the required volume of M-$NaH_2PO_4$. Distilled water, further purified upon a deionizer (Elgastat, Elga Products Ltd., London, S.W. 19), was used in all experiments.

*Radioactive tracers.* These have been described by Hele & Finch (1960).

*Preparations of amino acid-activating fractions from ribonucleic acid-low fractions of rat liver*

*Method* 1. Young Wistar rats of about 100 g. (two to four animals for each preparation) were employed. The livers were cooled in ice, weighed and minced through a stainless-steel tissue press into 2·3 vol. of medium A (Keller & Zamecnik, 1956). All manipulations were carried out at 0–4°. The mince was homogenized in a glass homogenizer, fitted with a motor-driven Teflon pestle, for three up-and-down strokes, taking about 45 sec. The homogenate was centrifuged for 15 000*g* for 10 min. in the Spinco model L preparative ultracentrifuge. The supernatant was pipetted off, the heaviest particles being rejected, diluted with 0·66 vol. of medium A and then centrifuged again at 105 000*g* for 30 min. The supernatant was again pipetted off and the protein concentration estimated (Gornall, Bardawill & David, 1949). A freshly prepared solution of protamine sulphate, 10 mg./ml., was added during 5 min. with mechanical stirring, in the proportion of 1·5 mg. of protamine to each 100 mg. of supernatant protein (about 7·5 mg. of protamine/10 g. of liver). The precipitate formed was removed by centrifuging at top speed (approx. 25 000*g*) for 3 min. in the superspeed angle head no. 69404 (4 × 25 ml.) of the MSE Major (refrigerated) centrifuge. From the supernatant, a fraction was precipitated with $(NH_4)_2SO_4$–potassium acetate (ASAc fraction, Hele & Finch, 1960) by adding 0·5 ml. of a cold 10 M-potassium acetate solution in 20 mM-EDTA to each 10 ml., followed by the addition of $(NH_4)_2SO_4$, precooled to −15° (2·8 g./10 ml.). All preparations by method 1 employed $(NH_4)_2SO_4$ recrystallized from mM-EDTA. When almost all the salt had dissolved the ASAc fraction was obtained by centrifuging at approx. 25 000*g* as described above. The precipitate was dissolved in 20 mM-tris–HCl, pH 7·2 (5 ml./10 g. of liver). The yield of protein was about 120 mg./10 g. of liver.

These amino acid-activating enzymes, like the acetate-activating enzyme of ox-heart mitochondria (Hele, 1954), can be further purified by gels in the presence of $(NH_4)_2SO_4$. These fractions are designated as 'RNA-low fractions'. The ASAc fraction (undialysed) was diluted with cold water to give a protein concentration of 10 mg./ml. The $(NH_4)_2SO_4$ saturation (Dixon & Webb, 1958) was then about 2·5%. Slight variations of this value did not appear to affect the treatment with calcium triphosphate gel, nor did the age of the gel; 1 mg. of gel (dry wt.) was added for every 15 mg. of protein. The gel was stirred in as rapidly as possible, and the mixture centrifuged without further delay in the superspeed head by bringing the head to top speed and allowing it to slow down as soon as top speed was reached. This preliminary treatment with a small amount of gel was found to remove an inhibitor. This precipitate was discarded and a further quantity of gel added in the proportions 1·0 mg. of gel/1·5 mg. of the starting amount of protein, and the mixture centrifuged as before. The precipitate was eluted with 0·75 vol. of the original diluted ASAc fraction, a mixture of 3 parts of 0·1 M-sodium phosphate, pH 8·0, and 1 part of saturated $(NH_4)_2SO_4$, pH 6·5, being used. The gel was spun down, eluted with 0·5 vol. of the same elution mixture and the eluates were combined. 10 M-Potassium acetate was added, equivalent to 0·025 vol. of the combined eluates, followed by saturated $(NH_4)_2SO_4$ (0·25 vol. of the combined eluates). The final $(NH_4)_2SO_4$ saturation was approx. 40%. After 5 min. the precipitate was collected by centrifuging as before, and dissolved in medium A (Keller & Zamecnik, 1956) to give a protein concentration of 10–15 mg./ml. The $(NH_4)_2SO_4$ saturation of this RNA-low fraction was about 5% (approx. 250 mM). About 15–20 mg. of protein was obtained from 10 g. of liver.

*Method* 2. The cooled, weighed livers were minced into 3 vol. of medium C (Hele & Finch, 1960), stirred briskly for 2 min. and then filtered through two layers of cheesecloth. A freshly prepared solution of protamine sulphate (10 mg./ml.) was added to the filtrate (1·0 ml./10 g. of liver), the addition taking 2 min. with constant stirring. The bulk of the precipitated material was removed by centrifuging at 1880*g* for 10 min. and the supernatant further centrifuged at 105 000*g* for 10 min. The ASAc fraction was prepared as for method 1, but required an additional centrifuging at 105 000*g* for 10 min. to remove a little insoluble material. The yield of ASAc fraction was a little higher than with method 1, being about 140 mg. for 10 g. of liver. This differs from pig liver, where the yield of soluble protein from tissue-mince extracts was 50% of the yield obtained from homogenates (Hele & Finch, 1960). The ASAc fraction was further purified, as under method 1, and the final yield of protein in the RNA-low fraction dissolved in medium C was about 15–20 mg. for 10 g. of liver. All preparations by method 2 employed potassium acetate without EDTA and $(NH_4)_2SO_4$ which had been recrystallized from water.

The yield of total enzyme units (substrates: leucine, isoleucine and lysine) in the RNA-low fractions was much the same with either method, and could be in excess of 100% of the total units present in the ASAc fractions. The increase in specific activity was five- to ten-fold, and was probably due to removal of inhibitors and lowering of 'blank' reactions, as well as to concentration of enzyme protein. The yield of enzyme units from 10 g. of liver in the RNA-low fractions was comparable with the yield of enzyme units in pH 5·0 fractions, if the marked effects of $MgCl_2$ concentration and 'soluble' RNA on some of the reaction rates were taken into account.

The RNA-low fractions contained no RNA detectable by the method of Hele & Finch (1960). The extinction ratio of the fractions, 280 mμ:260 mμ, varied between 1·4 and 1·6.

With pig liver, Hele & Finch (1960) found it necessary to perform pilot runs to determine the correct amount of protamine needed to remove particles from the liver extracts. This was not necessary with rat liver, provided that freshly prepared solutions of protamine were employed. In some of the earlier experiments, with method 2, the protamine solutions used were stored at −15° and thawed just before use. Enzyme preparations made with these protamine solutions gave leucine-activating enzymes of higher specific activity (measured at 6 mM-$MgCl_2$) than those in which freshly prepared protamine solutions were employed. After about six cycles of freezing and thawing, these protamine solutions suddenly and unpredictably lost much of their ability to precipitate particles.

The use of potassium acetate in conjunction with $(NH_4)_2SO_4$ was retained, for, although no longer required as a stabilizer (Hele & Finch, 1960), better yields of enzyme units were obtained in its presence.

These procedures for obtaining RNA-low fractions were not devised with the object of achieving a systematic purification of the enzymes under investigation, but to

provide material that could be compared with pH 5·0 fractions. For this purpose it was necessary that the specific activities of the enzymes in the two types of preparation should be comparable, that the time consumed in isolating the two types of fraction was similar, and that the manipulations used should be of comparable duration and severity. For this reason dialysis procedures were avoided. In the experiments upon enzyme stability, $(NH_4)_2SO_4$ and potassium acetate were added to the pH 5·0 fractions for the preincubation tests so that the salt concentrations were similar to those in the RNA-low fractions.

No correlation was found between the RNA content of these preparations as measured by chemical or optical methods, and the behaviour of the enzymes in these preparations. Some preparations, made by method 2 with frozen and thawed protamine solutions that had lost much of their ability to precipitate particles, had extinction ratios, 280 mμ:260 mμ, of 0·75. Comparable ratios were obtained with pH 5·0 fractions, yet the enzymes in these preparations behaved as would be expected of enzymes in RNA-low fractions. The results obtained with ribonuclease-treated pH 5·0 fractions suggest that fragments of 'soluble' RNA, not detectable by simple chemical methods, can still influence pyrophosphate-exchange rates.

*Fractions precipitated at* pH 5·0. These were made as described by Hele & Finch (1960), by a procedure based on that of Keller & Zamecnik (1956). Usually, two to four animals were used for each preparation. The medium A employed was freshly prepared immediately before use, by the addition of solid $KHCO_3$ to a stock solution of all the other components of this medium.

*Ribonuclease-treated* pH 5·0 *fractions.* These fractions, dissolved in medium A and of protein concentration about 20·0 mg./ml., were incubated at 37° for 15 min. with 400 μg. of ribonuclease and 2 μmoles of EDTA/ml. These conditions destroyed all the RNA detectable as cold perchloric acid-precipitable ribose (Hele & Finch, 1960). The EDTA stimulated the action of ribonuclease upon the RNA of pH 5·0 fractions. This procedure was used only with the experiments on $MgCl_2$ concentrations.

*'Soluble' ribonucleic acid.* This was isolated as described by Hele & Finch (1960).

### *Assays of enzyme activity*

*Pyrophosphate exchange.* The standard assay was similar to that described by Hele & Finch (1960), except that 6 μmoles of sodium ATP were employed. Magnesium was added as $MgCl_2$ in the quantities indicated. To prevent the formation of precipitates at the higher concentrations, $MgCl_2$ was added to the other reagents after they had been diluted to the final volume. In some of the earlier experiments 6 mM-disodium magnesium ATP was used. In experiments with high concentrations of AMP it was necessary to add extra charcoal to adsorb both AMP and ATP. The assay for pyrophosphate exchange in this case consisted of a mixture of 0·5 μmole of amino acid, 3 μmoles of sodium ATP, 1·5 μmoles of [$^{32}$P]pyrophosphate, 50 μmoles of tris–HCl, pH 7·2, AMP, NaCl and $MgCl_2$ added as indicated in individual experiments, and 0·1–0·5 mg. of protein, in a final volume of 0·55 ml. Charcoal equivalent to 400 mg. dry wt. was used. Recovery of 65–75% of orthophosphate from ATP was obtained.

A number of pilot experiments were performed with fractions stored at −15°, with or without added GSH; but all definitive experiments were carried out with fractions tested upon the day of preparation.

A unit of enzyme activity is defined as that amount of enzyme which catalyses the incorporation of 1·0 μm-mole of [$^{32}$P]pyrophosphate into ATP in 1 min. under standard assay conditions. Since no single standard of $MgCl_2$ concentration can be defined, the $MgCl_2$ concentration employed will be stated where necessary for the individual enzymes. Specific activity is defined as enzyme units/mg. of protein. All rates for amino acid-dependent pyrophosphate exchange are given after subtraction of the appropriate amino acid-free blank even when the blank value is also given.

*Attachment of* [$^{14}$C]*amino acids to 'soluble' ribonucleic acid.* The procedure used has been described by Hele & Finch (1960). Where less than 1 mg. of protein was incubated with 'soluble' RNA an extra 2 mg. of protein, dissolved in a volume of 0·1–0·2 ml., was added immediately before stopping the reaction with cold perchloric acid. The recoveries of RNA in this instance were about 70% of the added RNA, but were as consistent amongst themselves as the recoveries (about 90%) obtained when the protein was present in the incubation from the start. The protein used was obtained from rat-liver or pig-liver fractions which had been treated with calcium triphosphate gel to remove activating enzymes. Such fractions were precipitated with $(NH_4)_2SO_4$ and dialysed for 18 hr. against 20 mM-tris–HCl, pH 7·2. This protein could not incorporate [$^{14}$C]amino acids into 'soluble' RNA.

*Measurement of radioactivity.* The procedures were carried out as previously described (Hele & Finch, 1960). All samples containing [$^{14}$C]amino acid were counted to give an accuracy of ±5% on the less radioactive samples (i.e. giving an incorporation of less than about 0·5 μm-mole/mg. of 'soluble' RNA). The accuracy on the more radioactive specimens was ±2–3%.

*Chemical estimations.* These were as described by Hele & Finch (1960).

## RESULTS

### *Influence of magnesium chloride concentration and 'soluble' ribonucleic acid upon amino acid-dependent pyrophosphate exchange*

The rates of leucine-, isoleucine- and lysine-dependent pyrophosphate exchange catalysed by pH 5·0 fractions showed a response to changes in $MgCl_2$ concentration that were highly characteristic for each amino acid (Fig. 1). The main features were observed with a number of pH 5·0 fractions, although some variation occurred in detail.

With isoleucine as substrate the maximum rate of pyrophosphate exchange was achieved between 10 and 20 mM-$MgCl_2$, and was between two and three times the rate measured with 5 mM-$MgCl_2$. It was highly characteristic of this enzyme that rates of this magnitude (relative to the rate at 5 mM-$MgCl_2$) were maintained between 10 and 30 mM-$MgCl_2$. With leucine as substrate the maximum rate of pyrophosphate exchange occurred at 5 mM-$MgCl_2$, and fell off sharply with further

increases in $MgCl_2$ concentration. With lysine as substrate, little pyrophosphate exchange could be detected with less than 10 mM-$MgCl_2$. Above this concentration, the rate rose rapidly until concentrations of 20–30 mM were reached.

In RNA-low fractions (Fig. 2*a*), the responses of leucine- and isoleucine-dependent pyrophosphate-exchange rates to changes in $MgCl_2$ concentration were remarkably similar. With isoleucine as substrate the maximum rate of pyrophosphate exchange occurred at 10 mM-$MgCl_2$, and fell off sharply with further increases in $MgCl_2$ concentration. With leucine as substrate the maximum rate of pyrophosphate exchange occurred at 15 mM-$MgCl_2$. With lysine as substrate the $MgCl_2$ concentration curve was similar to that observed with pH 5·0 fractions.

Addition of phenol-isolated 'soluble' RNA, made from fresh pH 5·0 fractions, resulted in $MgCl_2$ concentration curves for leucine and isoleucine very similar to those obtained with pH 5·0 fractions, but these curves (Fig. 2*b*) were not identical with those observed with pH 5·0 fractions.

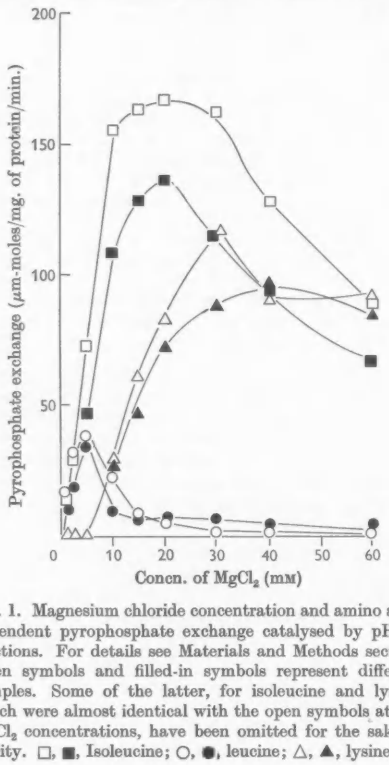


Fig. 1. Magnesium chloride concentration and amino acid-dependent pyrophosphate exchange catalysed by pH 5·0 fractions. For details see Materials and Methods section. Open symbols and filled-in symbols represent different samples. Some of the latter, for isoleucine and lysine, which were almost identical with the open symbols at low $MgCl_2$ concentrations, have been omitted for the sake of clarity. □, ■, Isoleucine; ○, ●, leucine; △, ▲, lysine.

The curve for lysine-dependent pyrophosphate exchange did not alter significantly in this experiment, which did not include $MgCl_2$ concentrations above 30 mM.

The pH 5·0 fractions, preincubated at 37° to remove the terminal nucleotide sequence of 'soluble' RNA (Hecht *et al.* 1959), showed significant alterations in the response of these enzymes to changes in $MgCl_2$ concentration at and above 10 mM (Fig. 3*b*). The ability of the leucine-activating enzyme to catalyse pyrophosphate exchange at high $MgCl_2$ concentrations increased, whereas that of the isoleucine-activating enzyme declined markedly, the rates with isoleucine at the higher concentrations being no longer two to three times the rate observed at 5 mM-$MgCl_2$, as was the case with fresh pH 5·0 fractions. With lysine as substrate the rate of increase of pyrophosphate exchange fell off at

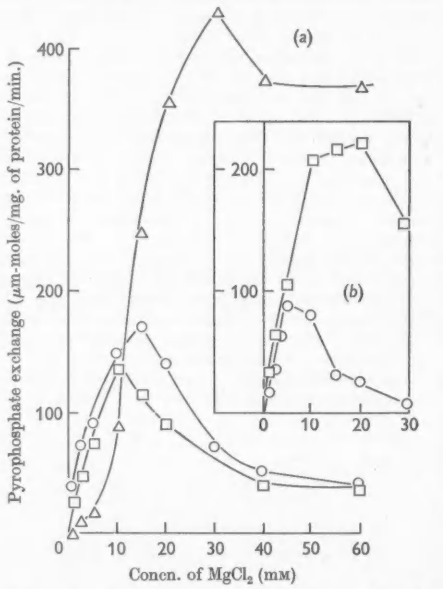


Fig. 2. Magnesium chloride concentration and amino acid-dependent pyrophosphate exchange catalysed by: (*a*) RNA-low fractions; (*b*) by such fractions with added 'soluble' RNA. □, Isoleucine; ○, leucine; △, lysine. For (*a*), the assay was as described under Materials and Methods, but for (*b*) it was scaled down to a total volume of 0·5 ml. The concentration of RNA-low fraction protein in the assay for (*a*) was 0·44 mg./ml. and for (*b*) was 0·76 mg./ml., that of 'soluble' RNA being 90 μg./ml. The RNA-low fraction was prepared by method A, and the 'soluble' RNA was isolated from a freshly prepared pH 5·0 fraction. Different enzyme preparations were used in the two experiments.

$MgCl_2$ concentrations above 15 mM, instead of continuing to rise fairly sharply, as found both with pH 5·0 fractions and RNA-low fractions. Similar modifications to the $MgCl_2$ concentration curves were observed (Fig. 3*a*) with ribonuclease-treated pH 5·0 fractions. These preparations contained no 'soluble' RNA detectable by simple chemical methods, and could not incorporate [$^{14}$C]amino acids into 'soluble' RNA.

The effects of $MgCl_2$ concentration upon the rates of pyrophosphate exchange were also investigated in the presence of other amino acids (Figs. 4*a* and *b*). For threonine and valine they were similar to that observed with lysine.

Hele & Finch (1960) reported that, relative to leucine, less [$^{14}$C]threonine and [$^{14}$C]valine were transferred to 'soluble' RNA in pH 5·0 fractions than in 'recombined' systems. The transfer of [$^{14}$C]lysine shows the same phenomenon (Tables 1 and 2). The best incorporations of [$^{14}$C]lysine into recombined systems were sometimes obtained with the smallest amounts of protein and short incubation periods.

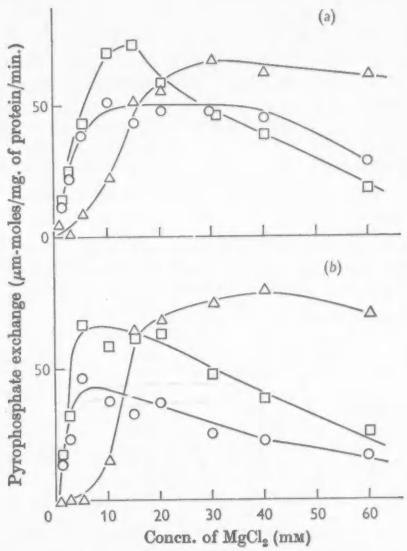


Fig. 3. (*a*) Effect of ribonuclease treatment on amino acid-dependent pyrophosphate exchange catalysed by pH 5·0 fractions at varying $MgCl_2$ concentrations. Details of the ribonuclease treatment are given under Materials and Methods. □, Isoleucine; ○, leucine; △, lysine. (*b*) Effect of preincubation on amino acid-dependent pyrophosphate exchange catalysed by pH 5·0 fractions at varying $MgCl_2$ concentrations. The pH 5·0 fraction (20 mg. of protein/ml.) was incubated at 37° for 30 min. and cooled in ice, before testing as described under Materials and Methods. The preincubated fraction contained 2·0 mg. of 'soluble' RNA/100 mg. of protein, but could only incorporate the following amounts of [$^{14}$C]amino acid into this 'soluble' RNA: isoleucine, 0·27; leucine, 0·23; lysine, 0·12 (μm-mole/mg. of 'soluble' RNA/4 min.). The assay for [$^{14}$C]amino acid incorporation is described under Materials and Methods. □, Isoleucine; ○, leucine; △, lysine.

### *Depression of amino acid-dependent pyrophosphate exchange by adenosine 5′-phosphate*

The effect of adding AMP upon amino acid-dependent pyrophosphate exchange was studied at four different $MgCl_2$ concentrations (Table 3). At the two higher $MgCl_2$ concentrations sodium AMP depressed the lysine-dependent pyrophosphate exchange to about half of that in the presence of a control addition of NaCl. This effect was observed with both pH 5·0 fractions and RNA-low fractions. Similar results were obtained with 36 mM-$MgCl_2$. Inorganic orthophosphate (13·5 mM, sodium salts, pH 7·2) was without effect. With leucine as substrate, similar results were obtained, but only when RNA-low fractions were used. The degree of depression produced by AMP at 27 mM-$MgCl_2$ is dependent upon the amount of AMP added (Fig. 5). Tris–AMP was somewhat more effective than

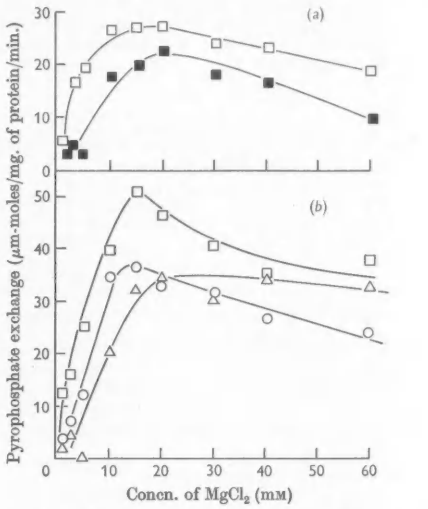


Fig. 4. (*a*) Concentration of $MgCl_2$ and amino acid-dependent pyrophosphate exchange catalysed by 'RNA-low' fractions. The standard assay system, described under Materials and Methods, was employed. □, Methionine; ■, valine. (*b*) Concentration of $MgCl_2$ and amino acid-dependent pyrophosphate exchange catalysed by pH 5·0 fractions. □, Methionine; ○, tryptophan; △, threonine.

sodium AMP, suggesting that $Na^+$ ions may counteract the AMP.

At the two lowest $MgCl_2$ concentrations used (Table 3), the effect of adding AMP was not reproducible. For example, in two experiments with RNA-low fractions, similar to that shown in the Table, the pyrophosphate-exchange rate, observed at 0·9 mM-$MgCl_2$ with and without leucine, was unaffected by the addition of 13·5 mM-sodium AMP. Leucine-dependent pyrophosphate exchange, catalysed by pH 5·0 fractions in the presence of 13·5 mM-NaCl, was greater at 13·5 mM-$MgCl_2$ than at 4·5 mM-$MgCl_2$. This change was not due to changes in the amino acid-free blank. This blank was more variable when pH 5·0 fractions were used than when RNA-low fractions were employed.

Table 1. *Incorporation of* [$^{14}$C]*leucine and* [$^{14}$C]*lysine into 'soluble' ribonucleic acid catalysed by* pH 5·0 *fractions*

The assay system described under Materials and Methods was used with samples (0·25 ml.) of pH 5·0 fractions. In Expt. 1 the $MgCl_2$ concentration was 30 mM; in Expts. 2 and 3 it was 10 mM. In all experiments the protein concentration was about 10 mg./ml.

| Expt. no. | Incubation time (min.) | [$^{14}$C]Amino acid incorporated into 'soluble' RNA ($\mu$m-moles/mg.) Leucine | Lysine |
|---|---|---|---|
| 1 | 4 | 3·2 | 1·0 |
| 2 | 2 | 4·5 | 1·8 |
| | 4 | 4·5 | 2·4 |
| | 10 | 5·0 | 2·5 |
| 3 | 4 | 3·3 | 1·9 |
| | 10 | 3·3 | 4·0 |

*Stability of the enzymes*

RNA-low fractions containing recrystallized $(NH_4)_2SO_4$ and preincubated before assay by keeping at 37° lost much of their capacity to catalyse leucine-dependent pyrophosphate exchange (Table 4). This loss was reduced by the addition of GSH or EDTA. The addition of yeast RNA, or of 'soluble' RNA from liver, in amounts equivalent to those present in pH 5·0 fractions, did not give protection.

The pH 5·0 fractions were very stable when kept at 37° under similar conditions (Table 5). The

Table 2. *Incorporation of* [$^{14}$C]*leucine and* [$^{14}$C]*lysine into 'soluble' ribonucleic acid catalysed by 'recombined' systems*

The assay system described under Materials and Methods was used. The final $MgCl_2$ concentration was 10 mM. Times in parentheses refer to the period of incubation. The RNA-low fraction was prepared by method B with a frozen and thawed solution of protamine. 'Soluble' RNA (113 $\mu$g.) was added to the assay in Expt. 1; 100 $\mu$g. was used in Expt. 2.

| Ribonucleic acid-low protein added (mg.) to 0·4 ml. of reaction mixture | Amino acid incorporated into 'soluble' RNA ($\mu$m-moles/mg.) Without GSH Leucine | Lysine | With 10 mM-GSH Leucine | Lysine |
|---|---|---|---|---|
| Expt. 1. 3·8 (10 min.) | 6·3 | 4·9 | 6·2 | 5·0 |
| 0·05 (2 min.) | 0 | 2·1 | 4·8 | 4·6 |
| 0·10 (0·5 min.) | 0·1 | 2·1 | 3·7 | 4·2 |
| Expt. 2. 1·1 (10 min.) | 3·3 | 2·8 | 5·7 | 3·5 |
| 0·05 (2 min.) | 0 | 2·9 | 4·0 | 4·5 |

Table 3. *Effect of magnesium concentration and adenosine 5′-phosphate on amino acid-dependent pyrophosphate exchange catalysed by ribonucleic acid-low and* pH 5·0 *fractions*

The assay system described under Materials and Methods was used. The RNA-low fraction was prepared by method B. Sodium AMP was employed, 7·5 $\mu$moles being added to the appropriate assays, and 7·5 $\mu$moles of NaCl were added to the controls. Figures in parentheses refer to the amino acid-free controls and apply to experiments with lysine as well as leucine. Final vol. 0·55 ml.

| $MgCl_2$ ($\mu$moles) | Pyrophosphate exchange ($\mu$m-moles/mg. of protein/min.) RNA-low fraction Leucine −AMP | Leucine +AMP | Lysine −AMP | Lysine +AMP | pH 5·0 fraction Leucine −AMP | Leucine +AMP | Lysine −AMP | Lysine +AMP |
|---|---|---|---|---|---|---|---|---|
| 0·5 | 23 (26) | 5 (28) | 0 | 0 | 14 (13) | 7 (11) | 0 | 0 |
| 2·5 | 77 (25) | 66 (19) | 15 | 7 | 19 (18) | 25 (9) | 3 | 1 |
| 7·5 | 147 (23) | 79 (31) | 131 | 64 | 37 (17) | 9 (11) | 51 | 24 |
| 15·0 | 82 (26) | 43 (20) | 246 | 116 | 10 (19) | 8 (13) | 65 | 26 |

$(NH_4)_2SO_4$ and potassium acetate present in these experiments prevented the loss of the terminal nucleotide sequence of the 'soluble' RNA, as is indicated by the ability of the 'soluble' RNA to undergo labelling with [$^{14}$C]amino acids (Table 6). The pH 5·0 fractions did not lose ability to catalyse leucine-dependent pyrophosphate exchange, when kept at 37° either with or without the addition of the salts. However, after incubation for 10–15 min. in the absence of salts they became unstable on addition of the salts (Table 5). This suggested that the stability of the enzyme was connected with the completeness of the terminal nucleotide sequence of the 'soluble' RNA. Inclusion of 10 mM-GSH, along with the salts, conferred a considerable degree of protection upon the enzymes. Ribonuclease-treated fractions were also unstable when tested in this system.

Lysine-activating enzymes present in pH 5·0 fractions were less stable than leucine-activating enzymes when kept at 37° with $(NH_4)_2SO_4$ and potassium acetate. Both enzymes became more unstable upon storage at −15° for a few days, as tested by keeping at 37° in the presence of these salts. The loss of stability of the leucine-activating enzyme was accompanied by a rise in specific activity, measured with 10 mM-$MgCl_2$, which could not be accounted for by changes in protein concentration. The lysine-activating enzyme was the more unstable enzyme of the two and there was no indication that 'soluble' RNA conferred protection upon this enzyme in these tests. The two enzymes were about equally unstable when RNA-low fractions were kept at 37° with $(NH_4)_2SO_4$ and potassium acetate. The lysine-activating enzyme in

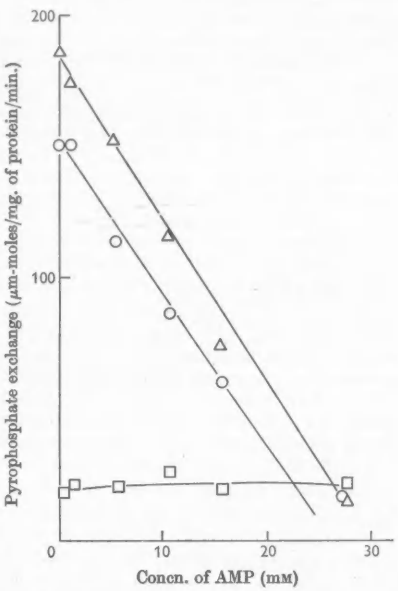


Fig. 5. Effect of tris–AMP on amino acid-dependent pyrophosphate exchange, catalysed by 'RNA-low' fractions. The assay system is described under Materials and Methods. Concentration of $MgCl_2$: 27 mM. The 'RNA-low' fraction was prepared by method B. ○, Leucine; △, lysine; □, no amino acids.

Table 4. *Effect of certain substances on the stability of the leucine-activating enzyme present in ribonucleic acid-low fractions*

The assay system described under Materials and Methods was used with 6 mM-disodium magnesium ATP. An additional 10 μmoles of $MgCl_2$ were added to the assays for the sample containing EDTA. The RNA-low fraction was prepared by method 2, with a frozen and thawed solution of protamine. This fraction was kept at 37°, at a protein concentration of approx. 5 mg./ml. for 30 min., and the recrystallized $(NH_4)_2SO_4$ concentration was about 100 mM The 0 min. samples were assayed after the additions had been mixed with the enzyme preparation but before keeping at 37°. The [$^{14}$C]leucine transfer to 'soluble' RNA amounted to 2·5 μm-moles/mg. of 'soluble' RNA/4 min. This was tested the same day, 1 mg. of RNA-low fraction protein and 115 μg. of 'soluble' RNA in a final volume of 0·4 ml. being used. No GSH was added to the assay.

| | Pyrophosphate exchange (μm-moles/mg. of protein/min.) | |
|---|---|---|
| Time of preincubation ... | 0 | 30 |
| Addition during preincubations | | |
| None | 156 | 42 |
| 100 mM-Tris–GSH | 174 | 144 |
| 'Soluble' RNA (188 μg./ml.) | 134 | 12 |
| Yeast RNA (135 μg./ml.) | 168 | 34 |
| 45 mM-EDTA | 140 | 120 |

Table 5. *Effect of preincubation on the stability of leucine-activating enzyme in* pH 5·0 *fractions*

The assay system described under Materials and Methods was used, with 6 mM-disodium magnesium ATP. Samples of pH 5·0 fraction were preincubated at 37° before assay at a protein concentration of about 5 mg./ml. No significant changes in any of the amino acid-free blanks were observed.

| | Leucine-dependent pyrophosphate exchange (μm-moles/mg. of protein/min.) | | |
|---|---|---|---|
| Time of preincubation (min.) | 0 | 15 | 40 |
| No addition | 42 | — | 44 |
| Salts added at 0 min. | 38 | — | 36 |
| Salts added at 15 min. | — | 41 | 26 |
| Salts plus GSH added at 15 min. | — | 45 | 40 |

Table 6. *Effect of certain substances on the ability of preincubated* pH 5·0 *fractions to incorporate* [$^{14}$C]*amino acids into 'soluble' ribonucleic acid*

Samples (0·2 ml.) of pH 5·0 fractions dissolved in medium A were preincubated in 15 ml. centrifuge tubes at 37° for 30 min. with the additions as indicated. The tubes were cooled in ice, and 0·02 ml. of N-$KHCO_3$ was added to those containing EDTA to prevent the formation of a precipitate when the other reagents were added. The final pH was not significantly changed (indicator paper). Magnesium chloride (6 $\mu$moles) and 4 $\mu$moles of ATP were added to all tubes, bringing the final volume to 0·4 ml., and the reaction was started by the addition of 0·04 $\mu$mole of [$^{14}$C]amino acid. Incubation was for 4 min. at 37°. Figures shown in parentheses in Expt. 2 were obtained from samples tested in the presence of the additions, but without preincubation.

| Additions to 0·2 ml. samples of pH 5·0 fraction | Amino acid incorporated into 'soluble' RNA ($\mu$m-moles/mg.) | |
|---|---|---|
| | Leucine | Lysine |
| Expt. 1 | | |
| No additions | 0·5 | 0 |
| Salts* | 3·7 | 1·6 |
| EDTA (2·5 $\mu$moles) | 3·8 | 0·9 |
| EDTA (0·25 $\mu$mole) | 1·1 | 0·3 |
| Not preincubated | 2·9 | 1·1 |
| Expt. 2 | | |
| No additions | 0·1 (3·2) | |
| Salts* | 3·6 (3·2) | |
| EDTA (5 $\mu$moles) | 2·6 (3·0) | |
| EDTA (0·5 $\mu$mole) | 0·6 (3·5) | |

* 26 $\mu$moles of recrystallized $(NH_4)_2SO_4$ and 5 $\mu$moles of potassium acetate.

pH 5·0 fractions and in RNA-low fractions was stimulated by keeping these preparations at 37° in the presence of 10 mM-GSH, whereas the leucine-activating enzyme was not significantly stimulated.

In other experiments in which pH 5·0 fractions and RNA-low fractions were treated with *p*-chloromercuribenzoate or kept at 37° in the presence of GSSG plus EDTA, the leucine-activating enzyme was more readily depressed than the lysine-activating enzyme (Fig. 6*a*, *b*). Omission of EDTA from experiments with pH 5·0 fractions enhanced the effect of GSSG upon the lysine-activating enzyme, but not that on the leucine-activating enzyme. Since EDTA prevents the loss of the terminal nucleotide sequence from 'soluble' RNA (Table 6) this suggests that the sequence protects the lysine-activating enzyme, but not the leucine-activating enzyme, from attack by GSSG. Alternatively, the lysine-activating enzyme is more sensitive than the leucine-activating enzyme to combined attack by traces of metals and GSSG.

The transfer of [$^{14}$C]leucine to 'soluble' RNA catalysed by 'recombined systems' (Hele & Finch, 1960) was considerably stimulated by the addition of 10 mM-GSH at low concentrations of protein. The transfer of [$^{14}$C]lysine was stimulated by GSH to a lesser extent under similar experimental conditions (Table 2). Addition of mM-EDTA to the reaction mixture, or treatment of the 'soluble' RNA preparation with mM-EDTA before addition to the reaction mixture, also protected the enzyme, suggesting that small amounts of heavy metals were bound to the 'soluble' RNA and acted as inhibitors (Goldstein & Holley, 1960) at low concentrations of protein.

## DISCUSSION

*Interaction of magnesium ions and 'soluble' ribonucleic acid in control of reactions catalysed by amino acid-activating enzymes*

The previous failure to detect an effect of added 'soluble' RNA upon the leucine- and isoleucine-pyrophosphate-exchange reactions (Hele & Finch, 1960) probably arose from the use of too little magnesium (6 mM) (Fig. 2*b*). The effects of 'soluble' RNA and higher concentrations of $Mg^{2+}$ ions are in opposite directions for the two reactions. At high magnesium concentrations the terminal nucleotide sequence of the 'soluble' RNA appears to be particularly important, as is shown by the changes in pyrophosphate-exchange rates after incubation of pH 5·0 fractions at 37° (Fig. 3*b*). Increased modification of the 'soluble' RNA, brought about by treatment with ribonuclease, results in further changes, particularly at magnesium concentrations of 10 mM and above. These modified pH 5·0 fractions give magnesium-concentration curves that do not fully resemble the curves observed with RNA-low fractions. In particular, the sharp maximum rate observed with leucine at 15 mM-magnesium chloride is not observed with the modified pH 5·0 fractions. This suggests that after removal of the terminal nucleotide sequence sufficient 'soluble' RNA remains behind to influence pyrophosphate-exchange rates, and that this is also true of the fragments of 'soluble' RNA resulting from ribonuclease treatment. These fragments are sufficiently small to escape detection as ribose, which is insoluble in cold perchloric acid.

These effects of different portions of the 'soluble' RNA molecules suggest that the pyrophosphate exchange may occur by two mechanisms. Perhaps one involves the formation of the amino acyl adenylate, and is controlled by the 'amino acid-specific' part of the 'soluble' RNA molecule (Schweet, Bovard, Allen & Glassman, 1958; Preiss, Berg, Ofengand, Bergmann & Dieckmann, 1959; Lipmann, Hülsmann, Hartmann, Boman & Acs, 1959); the other mechanism could involve amino acyl thioester intermediates and the terminal nucleotide sequence of the 'soluble' RNA (Hecht

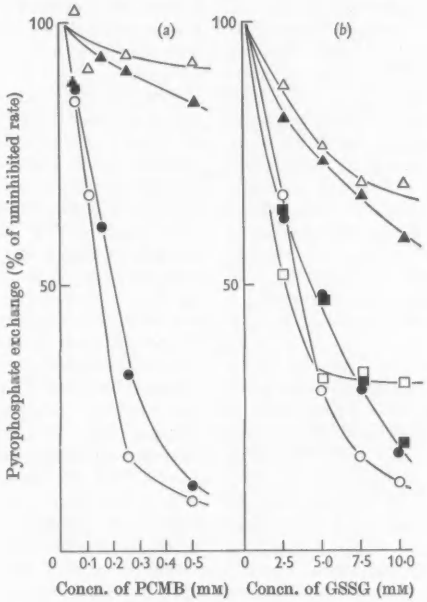


Fig. 6. (*a*) Effect of *p*-chloromercuribenzoate (PCMB) on amino acid-dependent pyrophosphate exchange. Samples of enzyme preparations, containing a final protein concentration of 10 mg./ml., were kept for 5 min. at 4° in the presence of the indicated concentrations of PCMB, before assay for pyrophosphate exchange. With lysine as substrate, the $MgCl_2$ concentration was 30 mM. With leucine, the $MgCl_2$ concentration was 5 mM with pH 5·0 fractions, and 15 mM with RNA-low fractions. The RNA-low fraction was prepared by method A. The 100% rates (symbols for which are omitted from the diagram) were obtained from samples, without PCMB, but otherwise treated in an identical manner. The values of these 100% rates (μm-moles/mg. of protein/min.) were as follows: pH 5·0 fraction with leucine (●) 46, with lysine (▲) 95, RNA-low fraction with leucine (○) 108, with lysine (△) 158. Addition of GSH (final concentration 20 mM) for a further 5 min. to samples of both fractions treated with 0·25 mM-PCMB gave complete restoration of all enzyme activities. (*b*) Effect of GSSG on amino acid-dependent pyrophosphate exchange. Samples of enzyme preparations, containing a final protein concentration of 10 mg./ml., were kept at 37° for 20 min. in the presence of 10 mM-EDTA and the indicated amounts of GSSG. After cooling in ice the samples were assayed as for Fig. 6 (*a*), except that 6 mM-$MgCl_2$ was used with the pH 5·0 fraction when leucine was used as substrate. EDTA (1 μmole) was added to each assay along with the samples of enzyme. The RNA-low fraction was prepared by method A. The 100% rates were given by samples that were preincubated at 37° for 20 min. in the presence of EDTA alone, and with the RNA-low fraction were identical with the rates obtained with a fresh sample. The values for the 100% rates (μm-moles/mg. of protein/min.) were as follows: pH 5·0 fraction with leucine (●) 38, with lysine (▲) 110, RNA-low with leucine (○) 141, with lysine (●) 266. In another experiment the EDTA was omitted and purified sucrose used in the composition of the medium A in which the pH 5·0 fraction was dissolved. In this experiment the pH 5·0 fraction gave these values for the 100% rate: with leucine (□) 41 and with lysine (■) 92.

*et al.* 1959; Canellakis, 1959). Alternatively, reactions priming the 'soluble' RNA molecule with ATP might be involved (Ogata, Nohara & Miyazaki, 1959).

Ogata, Nohara, Ishikawa, Morita & Asaoka (1961), using guinea-pig-liver preparations purified by chromatography on diethylaminoethylcellulose, have shown that the stimulating effect of 'soluble' RNA upon isoleucine-dependent pyrophosphate exchange does not require the terminal nucleotide sequence (Hecht *et al.* 1959); this is virtually the reverse of the observations made here. This discrepancy may do no more than reflect differences in experimental conditions, but other explanations should not be overlooked. The relatively unpurified pH 5·0 fractions may contain an additional factor which is required if the terminal nucleotide sequence is to exert its maximum effect upon pyrophosphate-exchange rates, and which might itself undergo partial destruction

during the incubation which removes the terminal nucleotide sequence of the 'soluble' RNA. The addition of 'soluble' RNA to RNA-low fractions does not restore completely the type of magnesium-concentration curve observed with pH 5·0 fractions (Figs. 1, 2*b*), especially at higher magnesium concentrations. Some of the factor may have been removed during the protamine treatment.

Under the experimental conditions used lysine-dependent pyrophosphate exchange is not markedly influenced by the presence or absence of 'soluble' RNA. The response to changes in magnesium concentrations, particularly at low concentrations, is the reverse of the response obtained with leucine and isoleucine.

The transfer of [$^{14}$C]lysine to 'soluble' RNA proceeds more rapidly with the 'recombined' systems than with pH 5·0 fractions. The transfer of [$^{14}$C]valine and [$^{14}$C]threonine was found to have this peculiarity by Hele & Finch (1960). The block in the transfer of these amino acids to the 'soluble' RNA of pH 5·0 fractions may be closely related to the fact that the effects of magnesium concentration upon the pyrophosphate-exchange reactions are similar for these three amino acids. Clearly, systems reconstructed from RNA-low protein and phenol-isolated 'soluble' RNA may not be identical with the original amino acid-activating system found in the pH 5·0 fraction.

AMP depresses leucine-dependent pyrophosphate exchange when this reaction is catalysed by RNA-low fractions at magnesium concentrations above 10 mM. At these concentrations the terminal nucleotide sequence of 'soluble' RNA exerts a predominant influence over the pyrophosphate-exchange rate. This suggests that the AMP may act by combining with a site upon the enzyme to which the 'acceptor' adenosyl unit of 'soluble' RNA is bound. Sodium ions counteract the effect of both AMP and 'soluble' RNA, probably by competing with $Mg^{2+}$ ions (Hamilton & Petermann, 1959). With the lysine-activating enzyme, AMP influences the pyrophosphate-exchange rate, but 'soluble' RNA does not. This may be connected with the block in the transfer of activated lysine to 'soluble' RNA.

*Labile groups of amino acid-activating enzymes*

The stability of the leucine-activating enzyme is related both to the manner in which 'soluble' RNA is associated with the enzyme in pH 5·0 fractions, and to the ability of the 'soluble' RNA to accept activated amino acid. The data suggest that the terminal nucleotide sequence of the 'soluble' RNA can protect a labile site associated with the enzyme. The experiments with AMP suggest that it may be the 'acceptor' adenosyl unit which confers this protection. The lysine-activating enzyme was not protected by 'soluble' RNA in the preincubation tests with ammonium sulphate and potassium acetate.

The relative degrees of deterioration, during storage, shown by the two enzymes are very similar to those reported by Allen, Glassman & Schweet (1960*b*) in pH 5·0 fractions prepared from guinea-pig liver. Stored pH 5·0 fractions retain their ability to transfer [$^{14}$C]amino acid to 'soluble' RNA for several weeks; therefore the change in the manner of association between 'soluble' RNA and enzyme that gives rise to the changes in specific activity and stability must be rather small and not involve any major destruction of the 'soluble' RNA. The protection conferred upon stored pH 5·0 fractions by GSH (Allen *et al.* 1960*b*) suggests that this change in the association between enzyme and 'soluble' RNA involves sulphydryl groups. The increase in specific activity of the leucine-activating enzyme, which occurs upon removal of 'soluble' RNA, and accompanies its loss of stability, resembles a similar association of changes observed with the alanine-activating enzyme present in ribonuclease-treated pH 5·0 fractions (Holley & Goldstein, 1959; Goldstein & Holley, 1960).

The effect of *p*-chloromercuribenzoate upon leucine-dependent pyrophosphate exchange is virtually the same with the rat-liver pH 5·0 fractions studied in this investigation and the guinea-pig-liver pH 5·0 fractions studied by Allen *et al.* (1960*b*). The high degree of resistance of the lysine-activating enzyme, as measured by pyrophosphate exchange, to treatment with *p*-chloromercuribenzoate is in striking contrast with its instability and its ability to be stimulated by GSH. Allen, Glassman, Cordes & Schweet (1960*a*) showed that the transfer of [$^{14}$C]lysine to added 'soluble' RNA catalysed by purified fractions obtained from guinea-pig liver was more resistant to treatment with *p*-chloromercuribenzoate than was the transfer of [$^{14}$C]leucine. This is consistent with my observations on the stimulatory effect of GSH upon these reactions.

The responses of the leucine- and lysine-activating enzymes to these activators and inhibitors recalls the individual type of response to inhibition with *p*-chloromercuribenzoate reported for the leucine- and threonine-activating enzymes of guinea-pig liver pH 5·0 fractions by Allen *et al.* (1960*b*). Conceivably, the effects may be correlated with their response to $Mg^{2+}$ ions and to 'soluble' RNA.

Allen *et al.* (1960*b*) suggested that sulphydryl groups might participate in the reaction mechanisms of amino acid activation. Perhaps the transfer of the activated amino acid to the adenosyl

unit involves a chelate mechanism and a thio ester intermediate (Hele, 1960*a*), along the lines suggested by Ingraham & Green (1958) for the activation of acetate.

## SUMMARY

1. The pyrophosphate-exchange reactions which are catalysed by rat-liver preparations and depend upon leucine or isoleucine are profoundly modified by 'soluble' ribonucleic acid and by changes in magnesium concentration. The preponderant influence is exerted by the terminal nucleotide sequence of the 'soluble' ribonucleic acid. Lysine-dependent pyrophosphate exchange occurs only at relatively high magnesium concentrations and is not greatly influenced by 'soluble' ribonucleic acid. The transfer of [$^{14}$C]lysine to 'soluble' ribonucleic acid is catalysed more rapidly by 'recombined' systems than by pH 5·0 fractions and may be related to the inability of the enzyme to react with low concentrations of magnesium, and to the presence of an inhibitor.

2. The terminal nucleotide sequence of 'soluble' ribonucleic acid may be concerned in the protection of a labile, essential site associated with the activating enzyme. The leucine- and lysine-activating enzymes show highly individual responses to activators and inhibitors of sulphydryl groups. The possible role of the labile site in the reaction mechanism is discussed.

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# A Phosphoprotein Phosphatase from Ox Brain

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It has been shown [see Heald (1959) for references] that the phosphorus of a phosphoprotein fraction of brain is exchanged rapidly when the tissue is stimulated *in vitro* by electrical pulses.

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During attempts to isolate this material (Heald, 1961*a*) it became apparent that brain contains an enzyme capable of splitting phosphate from phosphoprotein. Knowledge of such enzymes is limited and, apart from brief reports by Feinstein & Volk (1949) and Norberg (1950) that dispersions of rat

brain liberated phosphorus from unfractionated casein, no information was available about the existence of a cerebral phosphoprotein phosphatase. We have therefore extracted and partially purified a phosphoprotein phosphatase from ox brain and studied some of its properties. An account of part of this work was reported by Rose & Heald (1960).

## MATERIALS AND METHODS

*Casein.* α-Casein was prepared from commercial Hammarsten casein (Hopkin and Williams Ltd.) by fractionation with urea according to the method of Hipp, Groves & McMeekin (1955). β-Casein was a gift from Dr R. Aschaffenburg. The purity of the preparations was checked by electrophoresis at pH 8·6 in 0·04 M-sodium borate buffer, in a starch gel. Both caseins migrated as single and distinct components. The α-casein contained 0·92% of phosphorus (range 0·6–1·1%, six samples). The β-casein contained 0·36% of phosphorus.

*Phosvitin.* This was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., having been prepared by the method of Mecham & Olcott (1949). The dry product contained 7% of phosphorus, all of which was released as inorganic phosphorus on incubation in N-NaOH for 16 hr. at 37°. Before use it was dissolved in 10 mM-sodium ethylenediaminetetra-acetate (EDTA), pH 7·4, dialysed first against two changes of 1·0 mM-EDTA (100 vol.) for 24 hr. and finally for at least 48 hr. against four changes of water (100 vol.). Radioactive phosvitin was prepared by incubating 10 mg. of phosvitin (after dialysis) with radioactive phosphate in the presence of rat-liver mitochondria prepared and used as described by Burnett & Kennedy (1954). After incubation, usually for 30 min., the mitochondria were removed by centrifuging and the radioactive phosvitin was isolated by precipitation of the manganous salt as described by Rabinowitz & Lipmann (1960). The preparation was freed from manganese by dialysis against EDTA and water as described by these authors.

*Ovalbumin.* This was a twice-recrystallized sample from Nutritional Biochemicals Corp., and was salt-free.

*Pepsin and hexokinase.* These were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. The pepsin was a threefold-recrystallized specimen. The hexokinase was crude type II prepared from yeast.

*Casein phosphopeptones.* These were prepared by the digestion of α-casein with crystalline pepsin by the method of Damodaran & Ramachandran (1941). The yield from 1 g. of α-casein was 25 mg. of peptone containing 1% of phosphorus.

*Polyseryl phosphates.* These were obtained from a partial acid hydrolysate of phosvitin by electrophoresis on paper and elution of the materials separated (Heald, 1961*b*).

*Phosphoproteins from brain.* These were prepared as described by Heald (1961*a*).

*Sodium phosphoamidate.* This was a gift from Albright and Wilson Ltd., Birmingham. It was purified by solution in water below 10° and addition of acetic acid dropwise to pH 4·0, when the monosodium salt was precipitated. The precipitate was washed with water and ether and air-dried. It was reconverted into the disodium salt by the addition of the stoicheiometric amount of NaOH and the solution was adjusted to pH 6·0 before use. This method of purification (cf. Stokespre, 1893, 1894) was communicated by Albright and Wilson Ltd.

*Calgon PT, 'sodium tripholyphosphate' and 'phosphate glass 696'.* These polymetaphosphates were gifts from Albright and Wilson Ltd. (Alkaline Phosphates Division, Knightsbridge Green, London).

*Radioactive phosphate.* This was obtained as carrier-free inorganic orthophosphate from The Radiochemical Centre, Amersham, Bucks.

### *Analytical methods*

*Inorganic phosphate.* This was normally determined by the method of Martin & Doty (1949), modified for use with smaller samples. Thus the sample containing 1–15 μg. of phosphorus was transferred to a 15 ml. stoppered tube and brought to 3 ml. with water. Silicotungstate reagent (2 ml.) was added, followed by ammonium molybdate reagent (2 ml.) and *iso*butanol–benzene (1:1, v/v) (7 ml.). The mixture was shaken vigorously and centrifuged, and 5 ml. of the clear upper layer was mixed with stannous chloride reagent (1 ml.). The volume was brought to 10 ml. with ethanol–sulphuric acid (50:1, v/v). The extinction was read at 700 mμ against an ethanol–sulphuric acid blank. In the presence of labile phosphate esters inorganic phosphate was determined by the method of Lowry & Lopez (1946) as modified by Peel, Fox & Elsden (1955).

*Total phosphate.* This was determined as inorganic phosphate after digestion of the sample with perchloric acid (King, 1932).

*Determination of radioactive phosphate.* This was determined by counting the final *iso*butanol–benzene solutions used for the determination of inorganic phosphate in an M-6.H liquid counter (20th Century Electronics Ltd.).

*Nitrogen.* This was estimated, after digestion of samples by the method of Chibnall, Rees & Williams (1943), by distillation in the apparatus of Markham (1942).

*Phenol.* This was determined by the method of Folin & Ciocalteu as described by Snell & Snell (1953).

*Enzyme assay.* Appropriate quantities of the enzyme solution were incubated with α-casein (1·0 ml.; 4%, w/v) and sodium succinate buffer (0·1 M, pH 5·9). The final volume was 3·0 ml. After 15 min. at 37·5° the reaction was stopped by the addition of 2 ml. of 50% (w/v) trichloroacetic acid. After being chilled in ice for 20 min. the suspension was centrifuged and appropriate quantities were removed for the determination of inorganic phosphate. The large amount of trichloroacetic acid was necessary to ensure a complete precipitation of the α-casein. For the measurement of activity against various substrates (Tables 3 and 4; Figs. 5–7), the reaction mixture contained the enzyme (100–150 units), magnesium chloride (final concentration 0·3 mM), the substrate and sodium succinate, pH 5·9 (0·1 M), to a total volume of 3·0 ml.

*Units.* One unit of activity is defined as the amount of enzyme liberating 1 μg. of phosphorus/hr. from α-casein under the above-described conditions. Specific activity is defined as the number of units per mg. of nitrogen.

### *Purification procedure*

*Stage I: drying with acetone.* Ox brain was obtained from the Metropolitan Cattle Market as soon as possible after death of the animal and was packed in a polythene bag surrounded by ice during transport to the laboratory.

After removal of membranes and adhering blood clots, the brain was dispersed for 3 min. in 10 vol. of acetone at $-10°$ in a top-drive macerator. Suspensions were rapidly filtered with suction, washed with a further 3 vol. of acetone and finally with 3 vol. of diethyl ether. The powder was air-dried by suction and stored at $-18°$. Under these conditions no loss of enzymic activity was apparent for up to 10 weeks. Longer storage led to an appreciable loss of activity.

*Stage II: extraction of the enzyme.* The enzyme was extracted by grinding the powder, at room temperature, in a glass tube with a fitting pestle, with ten times its weight of water. The suspension was centrifuged for 15 min. at 78 400*g* and the supernatant retained. The residue was again extracted with a similar volume of water and after centrifuging for 30 min. at 78 400*g* the supernatant was removed. The combined supernatants constituted the 'water extract', in which the enzyme remained stable at 2° for up to 3 weeks.

*Stage III: acetone fractionation.* The water extract was chilled to 0° and stirred. Acetone (Hopkin and Williams Ltd., A.R.) at $-10°$ was slowly added to a final concentration of 50% (v/v) the temperature of the mixture being lowered to $-4°$ during the addition. For a typical preparation involving 200 ml. of the water extract the acetone was added over 40 min. The suspension was immediately centrifuged, at $-5°$, for 20 min. at 1500–1700*g* and the precipitate was discarded. The acetone concentration of the supernatant at $-5°$ was raised to 55% (v/v) and the mixture centrifuged for 20 min. at 1500–1700*g*. The small inactive precipitate was discarded. The concentration of acetone in the supernatant was then rapidly raised to 90% (v/v) and the mixture kept for 1 hr. at $-10°$ before being centrifuged for 20 min. at 1500–1700*g*. The precipitate was dissolved in one-fifth of the initial volume of water and stored at 2°. At this stage the enzyme retained full activity for up to 14 days.

*Stage IV: absorption on calcium phosphate gel.* The concentration of orthophosphate in the enzyme solution was adjusted to 700 $\mu$g./ml. by the addition of 0·1 M-$KH_2PO_4$. At this stage the pH of the solution was 6·4–6·6. Calcium chloride (0·1 M), equivalent to the total inorganic phosphate present, was then added with stirring. Stirring was continued for a further 18 hr. at 4° and the suspension was centrifuged. The supernatant was discarded.

*Stage V: elution from the gel.* The precipitate was shaken for 30 min. at 4°, first with a volume of tris (0·5 M, pH 7·4) equal to that of the solution at the beginning of stage IV. After centrifuging, the supernatant was discarded. The precipitate was shaken with a similar volume of sodium acetate (0·1 M, pH 6·4) for 30 min. at 4° and centrifuged. The precipitate was then shaken for 30 min. with a similar volume of sodium succinate buffer (0·1 M, pH 5·9) and centrifuged. This supernatant contained the enzyme purified some 90–100-fold over the original brain dispersion, in a yield of 20–25%. The overall purification and yields are summarized in Table 1.

## RESULTS

*Stability to fractionation.* Fractionation of the water extracts by means of ammonium sulphate, added either stepwise or rapidly to near saturation, led to losses of 60–100% of the enzymic activity. Similar losses also occurred during fractionation with acetone though not if the enzyme was precipitated rapidly at a final concentration of acetone of 90% (v/v). A product of high specific activity was obtained first by adding acetone slowly to a concentration of 55% (v/v), discarding the precipitate and rapidly adding more acetone to a final concentration of 90% (v/v). Unless the first addition was slow and the second rapid a considerable loss in activity occurred. This procedure increased the purity about 10–12-fold but the product completely lost its activity within 5 days at 2°. A less pure product which retained activity for at least 2 weeks was obtained by the procedure detailed in the Materials and Methods section and in Table 1. In all experiments with both ammonium sulphate and acetone, activity once lost was not restored by recombination of any or all of the fractions nor by the addition of cysteine or thioglycollate.

*Stability to dialysis.* The enzymic activity of the water extract was almost completely lost on dialysis against water at 0–4°; 50% of the loss occurred within the first 30 min. of the dialysis (Fig. 1). Recombination of the dialysis residue and the diffusate did not restore activity. The loss was

Table 1. *Purification of phosphoprotein phosphatase from ox brain*

Units are defined in the text. Percentages of acetone are by volume.

| Stage | Description | Volume (ml.) | Total units | Total N (mg.) | Specific activity | Recovery (%) | Purification factor |
|---|---|---|---|---|---|---|---|
| I | Aqueous acetone-dried powder | 210 | 35 280 | 656 | 54 | 100 | 1 |
| II | Water extract | 210 | 31 920 | 314 | 101 | 91 | 1·8 |
| III | 0–50% Acetone | 95 | 5 700 | — | — | 17 | — |
| | 50–55% Acetone | 50 | 0 | — | — | — | — |
| | 55–90% Acetone | 55 | 23 100 | 5·8 | 397 | 66 | 7·4 |
| V | Calcium phosphate gel eluates: | | | | | | |
| | pH 7·4 eluate | 50 | 0 | — | — | — | — |
| | pH 6·4 eluate | 50 | 2 500 | — | — | 7·0 | — |
| | pH 5·9 eluate | 35 | 6 650 | 1·3 | 5080 | 21·0 | 94 |
| | Gel residue | 25 | 2 000 | — | — | 6·0 | — |

not caused by contact with the dialysis tubing and was not prevented by dialysis against sodium chloride (0·1 M), magnesium chloride (0·1 M) or by the addition of bovine serum albumin (0·5 %) before dialysis. However, dialysis against 100 vol. of 20 mM-cysteine at pH 6·4–6·6 for 3 days did not reduce the activity of the enzyme. Cysteine did not prevent a total loss of activity during the dialysis of the more purified fractions from stages III and V.

pH *optimum and stability*. The effect of pH upon the activity and stability of the enzyme is shown in Fig. 2. The pH optimum of 5·5 lies below the pH of maximum stability and is in the region where α-casein becomes increasingly insoluble. For this reason the assay was performed at pH 5·9.

*Activators and inhibitors*. The effect of various ions upon the activity of the purified brain phosphoprotein phosphatase is presented in Table 2. Of the metals examined both magnesium and manganese markedly activated the brain enzyme. The optimum activation was obtained with 0·3 mM-$MgCl_2$. Higher concentrations were inhibitory (Fig. 3). Other metal ions were either without effect or inhibitory. As might be expected for an enzyme activated by magnesium, fluoride and EDTA were markedly inhibitory. The enzyme was not activated by thiols but was strongly inhibited by iodoacetate.

*Velocity and substrate concentration*. The enzyme from stage III was used to determine the velocity of phosphate release from different concentrations of α-casein in the absence of $Mg^{2+}$ ions (Fig. 4). A Lineweaver–Burk plot of the results gave $K_m$ 3·74 mg.atoms of α-casein phosphorus. Because of its low solubility the concentration of α-casein was limited to 10·0 mg.atoms of α-casein phosphorus/l.

*Activity towards various substrates*. At pH 5·9 the partially purified enzyme did not release phosphate from phenyl phosphate, β-glycerophosphate, glucose 6-phosphate, fructose 1:6-diphosphate, *O*-phosphorylserine, adenosine 5′-phosphate, sodium phosphoamidate, phosphocreatine, polyseryl peptides, Calgon PT, tripolyphosphate and phosphate glass '696' or a mixture of brain phospholipids. It did not act on diphenyl phosphate to yield phenol or phosphate. Some preparations of the enzyme occasionally released phosphate from adenosine 5′-triphosphate and sodium pyrophosphate. This was considered to be due to the presence of adenosine triphosphatase, which could be demonstrated by assaying under the conditions optimum for these two enzymes (Gore, 1951; Gordon, 1953) (Table 3).

*Activity towards other phosphoproteins*. The activity of the partially purified enzyme towards several phosphoproteins is shown in Table 4. The enzyme liberated phosphate from α- and β-casein and a derived peptone, phosvitin, ovalbumin and three preparations of brain phosphoprotein. No phosphate was released from yeast hexokinase or from pepsin. Failure to release phosphate from pepsin was not due to inactivation of the phosphatase since phosphate was released when α-casein was incubated in the system together with pepsin.

*Time-course of the release of phosphate*. Fig. 5 shows that the release of phosphate from α-casein

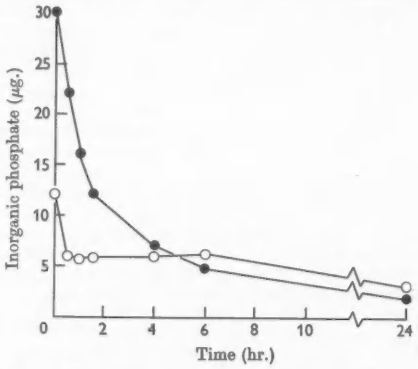


Fig. 1. Loss of phosphoprotein-phosphatase activity on dialysis against water. The water extract from stage II (see Materials and Methods section) at pH 6·4 was dialysed against water at 4°. Samples were removed periodically and assayed for activity in the system described in the Materials and Methods section. ○, Enzymic activity; ●, orthophosphate.

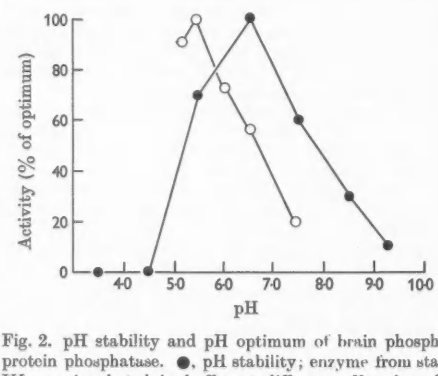


Fig. 2. pH stability and pH optimum of brain phosphoprotein phosphatase. ●, pH stability; enzyme from stage III was incubated in buffer at different pH values for 18 hr. at 2°, the pH adjusted to 5·9 and the enzyme assayed as described in the Materials and Methods section ○, pH optimum; the enzyme was assayed in buffers of varying pH. Buffer: pH 4·5–6·5, sodium succinate (0·2 M); pH 7·4–9·2, tris–HCl (0·1 M).

Table 2. *Effects of added substances on the activity of brain phosphoprotein phosphatase*

Reagents listed were tested in the system described in the Materials and Methods section, the enzyme from stage III or stage V being used. Superscript figures refer to the references listed below.

| Substance | Concn. (mM) | Activity (% of control) Brain enzyme | Enzymes from liver or spleen |
|---|---|---|---|
| $MgCl_2$ | 0·5 | 200 | 100[1] |
| $MgCl_2$ | 1·0 | 180 | 200[1, 3], 100[6] |
| $MnCl_2$ | 0·1 | 182 | 100[2], 150[1] |
| $CaCl_2$ | 1·0 | 100 | 150[1], 100[4, 5] |
| $CuSO_4$ | 1·0 | 90 | 40[5], 200[4], 50[6] |
| $ZnCl_2$ | 1·0 | 72 | 100[4], 50[6] |
| $CoCl_2$ | 1·0 | 27 | 100[1], 50[6] |
| $FeCl_2$ | 0·1 | 0 | 60[3], 400[4] |
| $MoO_4$ | 10·0 | 106 | — |
| $MoO_4$ | 0·1 | 95 | 0[4], 0[6] |
| NaF | 1·0 | 40 | 100[4], 82[6] |
| Iodoacetate | 10·0 | 12 | 70[6] |
| Iodoacetate | 1·0 | 38 | — |
| Iodoacetate | 0·1 | 47 | 100[3] |
| NaCN | 1·0 | 0 | 60[5], 150[1] |
| NaCN | 0·1 | 17 | — |
| EDTA | 1·0 | 25 | 100[6] |
| EDTA | 0·1 | 60 | — |
| Cysteine | 10·0 | 100 | 200–400[5], 275[3], 250[4] |
| Thioglycollate | 10·0 | 100 | 200–700[5], 200[3], 300[2] |
| Glutathione | 10·0 | 100 | — |
| Chloral | 0·3 | 100 | — |
| Phenobarbitone | 3·0 | 100 | — |

(1) Feinstein & Volk (1949); (2) Hofman (1958); (3) Singer & Fruton (1957); (4) Paigen & Griffiths (1959); (5) Sundararajan & Sarma (1954); (6) Revel & Racker (1960).

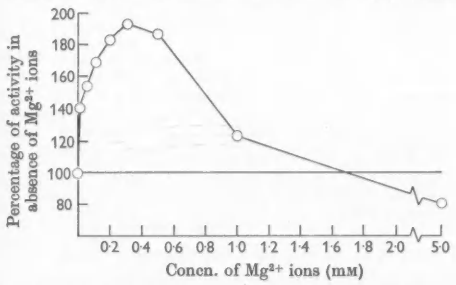


Fig. 3. Effect of increasing concentrations of $Mg^{2+}$ ions on the activity of brain phosphoprotein phosphatase. Enzyme from stage III. Experimental details were as described in the text.

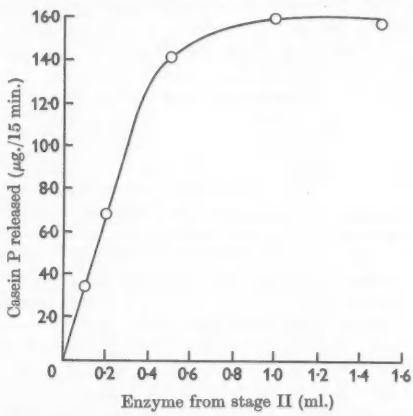


Fig. 4. Release of inorganic phosphate from α-casein with increasing concentrations of enzyme. The α-casein solution contained 10 mg.atoms of P/l. Enzyme from stage III. Incubation was for 15 min. at pH 5·9 and 37·5°.

was virtually complete within 20 min. in the absence of added $Mg^{2+}$ ions and was complete within this time in the presence of the ions. Cessation of activity was not due to loss of enzymic activity since the addition of fresh α-casein, after incubation for 60 min., resulted in an immediate release of more inorganic phosphate. Addition of fresh enzyme was without effect once the action had ceased showing that the phosphatase was inert towards the product of dephosphorylation. In the presence of $Mg^{2+}$ ions 85% of the available phosphate was liberated within 10 min. A higher rate of phosphate release was obtained with phosvitin; the release was complete in 4 min. and 75–80% of the available phosphate was liberated in 2 min.

This rate may not be maximal for phosvitin since release was measured under conditions optimum for α-casein; the optimum concentrations of $Mg^{2+}$ ions may not be the same for both proteins. With a concentration of magnesium of 0·3 mM the optimum concentration of phosvitin was that yielding a concentration of phosphorus of 4·0 μ-moles/ml. Higher concentrations of substrate inhibited the enzyme (Fig. 6), possibly owing to the binding of magnesium by excess of substrate. As with α-casein, once the reaction had ceased, the addition of further enzyme was without effect upon the dephosphorylated phosvitin.

*Phosphate release from radioactive phosvitin.* Cerebral tissues, yeast and liver are known to contain protein phosphokinases capable of phosphorylating added or intrinsic phosphoproteins (Burnett & Kennedy, 1954; Rabinowitz & Lipmann, 1960; Heald, 1961*a*). With a highly purified protein phosphokinase from yeast it was shown (Rabinowitz & Lipmann, 1960) that only a small fraction of the phosphorus from phosvitin was capable of metabolic exchange, though the actual amount could not be assessed. Phosvitin was made radioactive by phosphorylation with the protein phosphokinase in a rat-liver mitochondrial preparation (Burnett & Kennedy, 1954), and the product subsequently dephosphorylated by the partially purified phosphoprotein phosphatase. The time-course of the dephosphorylation is presented

Table 3. *Adenosine triphosphatase and inorganic pyrophosphatase in partially purified brain phosphoprotein phosphatase*

For the three substrates the assay systems were similar to those described in the Materials and Methods section and by Gore (1951) and Gordon (1953) respectively. Final concentrations: adenosine triphosphate and inorganic pyrophosphate, 7 mM; $Mg^{2+}$ ion, 20 mM. For pH 7·4 the buffer was 0·1 M-tris–HCl.

| | Phosphate released (μg. of P/15 min.) | | |
|---|---|---|---|
| pH | α-Casein | ATP | Inorganic pyrophosphate |
| 5·9 | 27·0 | 5·0 | 1·0 |
| 7·4 | 7·0 | 35·0 | 5·0 |

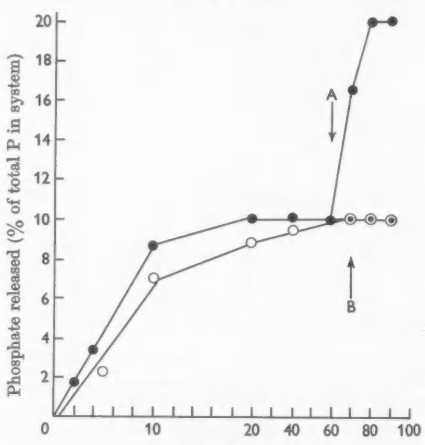


Fig. 5. Time-course of the release of inorganic phosphate from α-casein by the purified brain phosphoprotein phosphatase. System was as described in the Materials and Methods section. Enzyme from stage V. ○, Phosphate release in the absence of $Mg^{2+}$ ions; ●, phosphate release in the presence of 0·3 mM-$Mg^{2+}$ ions. Fresh α-casein was added at A to one set of experimental flasks; fresh enzyme was added at B to the other set of experimental flasks.

Table 4. *Activity of phosphoprotein phosphatase from brain upon phosphoproteins*

Activity was assayed as described in the Materials and Methods section with the enzyme purified to stage V. All substrates except brain phosphoprotein were used in concentrations to yield 1–4·0 μmoles of P/ml. of assay system. Brain phosphoprotein was used at a concentration of 0·3 μmole of alkali-labile P/ml. of assay system.

| | | | Phosphate released | |
|---|---|---|---|---|
| Phosphoprotein | Total P (%) | Alkali-labile P (%) | Percentage of total P | Percentage of alkali-labile P |
| α-Casein | 0·6 | 0·35 | 11 | 18 |
| β-Casein | 0·36 | 0·27 | 20 | 27 |
| Phosvitin | 7·0 | 7·0 | 5 | 5 |
| Ovalbumin | 0·09 | 0·09 | 6 | 6 |
| Hexokinase | 0·27 | — | 0 | — |
| Pepsin | 0·075 | 0·07 | 0 | 0 |
| α-Casein phosphopeptone | 0·8 | 0·50 | 17 | 26 |
| Brain phosphoprotein | | | | |
| I | — | 0·05 | — | 7·0 |
| II | — | 0·05 | — | 8·5 |
| III | — | 0·035 | — | 7·0 |

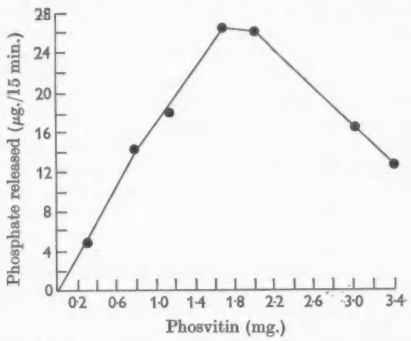


Fig. 6. Inhibition of enzymic activity by increasing concentrations of phosvitin when added to the enzyme in the presence of 0·3 mM-$Mg^{2+}$ ions. Enzyme from stage V.

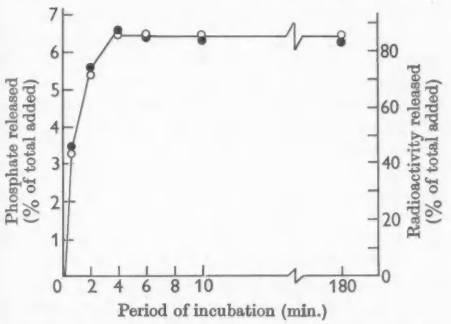


Fig. 7. Time-course of the release of radioactive phosphate from phosvitin phosphorylated in the system of Burnett & Kennedy (1954) and incubated with phosphoprotein phosphatase, as described in the Materials and Methods section. Enzyme from stage III. ○, Total phosphorus released; ●, radioactive phosphorus released.

in Fig. 7. The release of radioactivity paralleled that of inorganic phosphate and when the reaction has ceased 85 % of the total radioactivity had been released.

## DISCUSSION

The enzyme liberating inorganic phosphate from α-casein which has been prepared from ox brain can reasonably be described as a phosphoprotein phosphatase. In the absence of added $Mg^{2+}$ ions the rate of liberation of phosphorus from α-casein by aqueous dispersions of ox brain was 25–30 μmoles/g. wet wt. of tissue/hr. and, for similar dispersions of rat and guinea-pig brain, 50–60 μmoles/g. wet wt. of tissue/hr.

The pH optimum of 5·5–6·0 and the Michaelis constant of 3·7 mM-α-casein phosphorus are similar to the values found by Sundararajan & Sarma (1954) and Hofman (1958) for phosphoprotein phosphatase from spleen. In other respects, the two enzymes differ. The spleen enzyme, though purified to a state of homogeneity as determined by electrophoresis, ultracentrifuging and chromatography (Glomset, 1959; Glomset & Porath, 1960), was highly active towards phenyl phosphate, phosphocreatine, sodium phosphoamidate, inorganic pyrophosphate and adenosine triphosphate at pH 5·9. The brain-enzyme preparation did not act on the first four of these substrates and its slight activity towards the last-named two was probably due to the contaminating adenosine triphosphatase and inorganic pyrophosphatase (Table 3).

There are also sharp distinctions in the effects of activators and inhibitors on the two enzymes (Table 3). Thus the brain enzyme is activated by $Mg^{2+}$ and $Mn^{2+}$ ions but not by thiol compounds, whereas the spleen enzyme, reported variously to be activated or to be unaffected by $Mg^{2+}$ and $Mn^{2+}$ ions, has in all cases been stated to be activated by thiol compounds or ascorbate and in some cases to be inactive in the absence of thiols (Sundararajan & Sarma, 1957). In this respect the phosphoprotein phosphatase of rat or mouse liver (Feinstein & Volk, 1949; Paigen, 1958) appears to resemble more closely the spleen enzyme, being activated by thiols and reducing agents. Ferrous salts in concentrations up to 0·5 mM activate the phosphoprotein phosphatase of mouse liver (Paigen, 1958; Paigen & Griffiths, 1959) and of a stable spleen preparation (Revel & Racker, 1960), and 0·05 mM-molybdate strongly inhibits. The brain enzyme was inhibited by 0·1 mM-ferrous salts and not by molybdate at concentrations up to 10 mM. Unlike the stable spleen enzyme, that from brain was inhibited by sodium fluoride.

Brain phosphoprotein phosphatase is also less stable than this spleen enzyme, and in this respect the brain enzyme more closely resembles other phosphoprotein phosphatases obtained from ox spleen by Roche, Van Thoai & Pin (1953) and from chick embryo by Foote & Kind (1953). Both these enzymes were unstable in aqueous solution whereas the spleen enzyme was also inactivated when precipitated by ammonium sulphate. These last-named enzymes are distinct from the spleen preparations studied by Sundararajan & Sarma (1954), Hofman (1958), Singer & Fruton (1957) and Revel & Racker (1960), which are stable to dialysis and precipitation by ammonium sulphate and can be obtained by autolysis of spleen at pH 3·0 for 16 hr. In its instability, the brain enzyme resembles cerebral adenosine triphosphatase and inorganic pyrophosphatase (Gore, 1951; Gordon, 1953). These enzymes were also largely inactivated by dialysis or during precipitation with ammonium

sulphate, but their activities could be partially restored by incubation with cysteine.

Where comparable data exist the brain enzyme resembles in specificity at least the phosphoprotein phosphatase isolated from chick embryo by Foote & Kind (1953), or that studied in frog eggs by Harris (1946). Little additional information about the last-named two enzymes exists.

A further difference between the cerebral enzyme and that from spleen lies in its action upon the phosphoproteins α-casein and phosvitin. Thus the spleen enzyme liberates phosphate from casein or phosvitin over a period of several hours until the total phosphate has been removed (Sundararajan & Sarma, 1954; Hofman, 1958; Rabinowitz & Lipmann, 1960), whereas the brain enzyme rapidly liberates a definite amount of phosphate and then ceases to act. This phosphate includes most of the radioactive phosphorus incorporated by the action of a protein-phosphokinase preparation. This suggests that the brain phosphatase and the liver protein phosphokinase may be acting upon similar sites in the phosphoprotein molecule, but further work with more purified enzymes is needed. Phosvitin, as prepared by the method of Mecham & Olcott (1949), contains 25–30 phosphorus atoms/molecule. The release of 5–6% of the total phosphorus by the phosphatase corresponds to the release of approximately a single phosphate group/molecule.

The phosphoprotein phosphatase did not act on pepsin, which has a single seryl phosphate in the sequence glutamic acid–serine–phosphorus–threonine (Flavin, 1954). On the other hand, both casein and phosvitin contain sequences of adjacent seryl phosphate residues (Williams & Sanger, 1959; Heald, 1961*b*) and it has been suggested (Williams & Sanger, 1959; Rabinowitz & Lipmann, 1960) that sequences of this type are responsible for the ability of these phosphoproteins to act as acceptors for protein phosphokinases. It seems significant that the substrates attacked by the phosphatase are those capable of acting as phosphate acceptors for the protein phosphokinases.

The release of phosphate from ovalbumin is not understood. Ovalbumin has been found to be attacked by spleen phosphoprotein phosphatase (Singer & Fruton, 1957) and to act, to a slight degree, as a phosphate acceptor for the liver protein phosphokinase (Burnett & Kennedy, 1954). Ovalbumin contains two phosphorus groups/molecule and studies upon phosphopeptides derived from partial acid hydrolysates (Flavin, 1954) have established the existence of two different sequences containing seryl phosphorus. Possibly the effects noted above are due to impurities in the preparation. The phosphoprotein phosphatase from spleen has been used previously in attempts to investigate the nature of phosphate bonds in casein (Hofman, 1958) and has been studied as a possible means of detecting phosphorylated intermediates formed during oxidative phosphorylation (Revel & Racker, 1960). Such uses cannot yet be envisaged for the phosphoprotein phosphatase from brain, for though this enzyme is more specific than that from spleen, in the absence of further studies no firm conclusions can be drawn about the structures on which it acts.

## SUMMARY

1. A phosphoprotein phosphatase acting on α-casein has been purified from ox brain 100-fold and in a yield of 25%.

2. The pH optimum of the enzyme is 5·5. It is activated by $Mg^{2+}$ or $Mn^{2+}$ ions but not by thiol compounds. In the absence of $Mg^{2+}$ ions $K_m$ = 3·7 mg.atoms of α-casein phosphorus/l.

3. The enzyme lost activity when dialysed against water or salt solutions. With crude preparations the loss was prevented by dialysis against cysteine (20 mM). With more purified preparations cysteine did not prevent the loss of activity on dialysis. Activity was also lost during fractionation with ammonium sulphate and with graded concentrations of acetone.

4. At pH 5·9 the enzyme was inactive towards aliphatic and aromatic phosphonoesters, phosphoamides, a phosphodiester, inorganic polymetaphosphates, pyrophosphates, polyseryl phosphates and mixed phospholipids. It was active towards α- and β-casein, phosvitin, ovalbumin and preparations of brain phosphoprotein but not towards hexokinase or pepsin. Phosphopeptones prepared from α-casein were attacked by the enzyme.

5. The enzyme liberated only a small proportion of the total phosphate from α-casein and phosvitin before action ceased. The phosphorus released from phosvitin included 85% of the radioactive phosphorus incorporated by incubation with a protein phosphokinase.

6. The brain enzyme differs from phosphoprotein phosphatases derived from spleen or liver.

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# Enzymic Synthesis of 3:6-Anhydro-L-Galactose within Porphyran from L-Galactose 6-Sulphate Units

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3:6-Anhydrogalactose is of general occurrence as a constituent of the mucilaginous intercellular polysaccharides of the red seaweeds (Rhodophyceae), sometimes occurring as the D- and sometimes as the L-enantiomorph (for a review, see Araki, 1958). It was first discovered in commercial agar (Hands & Peat, 1938), and has been found in an increasing number of these polysaccharides (Yaphe, 1959). Jones & Peat (1942) pointed out that it could arise from the L-galactose 6-sulphate units which they postulated to be present in agar, perhaps by chemical action during the processing of the polysaccharide, since it had been shown that methyl galactopyranoside 6-sulphate when heated with alkali gave rise to methyl 3:6-anhydrogalactopyranoside (Duff & Percival, 1941). This view was contested (Barry & Dillon, 1944; Percival, 1944; Araki, 1953), because agar had never been isolated containing sufficient ester sulphate or L-galactose to account on this basis for the high anhydrogalactose content, and also because agar was not oxidized by the periodate ion. Percival (1944) pointed out that, if the precursor of the anhydro sugar is a derivative of a sugar sulphate, it need not be that of a 6-sulphate, since a sugar 3-sulphate could equally well be imagined to give 3:6-anhydrogalactose on suitable treatment. A further mechanism was proposed by Araki (1953), who suggested that oxidation at C-6 and reduction at C-1 would convert D-galactofuranose into 3:6-anhydro-L-galactose.

Hirst (1958) has again drawn attention to the possible role of galactose sulphate derivatives as precursors, but leaving open the nature of the derivatives involved. The interconversion and modification of monosaccharides usually occurs at the sugar nucleotide or sugar phosphate level (Hough & Jones, 1956; Hassid, Neufeld & Feingold, 1959). However, the possibility that this transformation from L-galactose 6-sulphate into 3:6-anhydro-L-galactose, if it occurs, takes place at the polysaccharide level, cannot be excluded, because the reaction might take place before the polysaccharide is deposited in the intercellular regions of the organism, and the fact that it was once highly sulphated would not then be evident (Percival, 1949).

Porphyran, the major polysaccharide of the red seaweed *Porphyra umbilicalis*, is related to agar in that it contains residues of 3:6-anhydro-L-galactose (10·3%; Peat, Turvey & Rees, 1961). It also contains residues of L-galactose 6-sulphate (Turvey & Rees,

1961). This paper shows that these latter units in the polysaccharide can be converted into 3:6-anhydrogalactose units by the action of an enzyme partially purified from extracts of the parent seaweed. A preliminary account of this work has been published (Rees, 1961*a*).

## METHODS

*Viscometric detection of depolymerase activity.* This was carried out in a thermostat at 35° in an Ostwald viscometer with a flow time of 32·5 sec. for water.

*Turbidimetric determination of liberated sulphate.* This was carried out as described by Rees (1961*b*).

*Estimation of* 3:6-*anhydrogalactose in enzyme digests.* This procedure is based on that of Yaphe (1960). Portions of incubation mixture (0·05–0·50 ml. containing 10–80 $\mu$g. of 3:6-anhydrogalactose) were mixed in test tubes (6 in. × 1 in.) with resorcinol–hydrochloric acid solution (10 ml.). This latter reagent was prepared immediately before use by mixing hydrochloric acid (sp.gr. 1·18; 200 ml.), resorcinol (0·13% in ethanol; 20 ml.) and water (40 ml.). The tubes were placed in a water bath at 80° (10 min.) and then in cold water (5 min.). The resulting crimson solutions were measured in an EEL photoelectric colorimeter, with filter no. 623 (maximum transmission at 495 m$\mu$). The amount of anhydro sugar present was calculated by reference to a standard curve, which was obtained by calibrating the reagents with porphyran of known (10·3%) 3:6-anhydrogalactose content. This standard material was analysed in the first instance by a modification (Peat *et al.* 1961) of the absolute method of O'Neill (1955), which depends upon estimating the hydroxymethylfurfuraldehyde produced by acid degradation of the anhydro sugar.

*Incubation of enzyme with substrates.* The sample of porphyran used was the same as that used by Peat & Rees (1961) unless otherwise stated. As low a substrate concentration as possible was used, since the detection of enzyme activity depended on the estimation of the difference between the 3:6-anhydrogalactose originally present and that present after a suitable incubation period. Two methods of incubation were therefore used. The first was as described by Rees (1961*b*): the mixture contained porphyran (1%, w/v, in water; 1 ml.) together with freeze-dried enzyme (0·05–0·2 mg.) and the specified electrolyte (0·5 ml.). The incubation period was normally 90 min. Since in this method the enzyme was saturated with substrate, it was used for 'quantitative assay' of enzyme. Small portions (0·05 ml.) of the digest were withdrawn in quadruplicate for treatment with the resorcinol–hydrochloric acid reagent. The second method ('semi-quantitative assay') was devised in order to obtain higher readings on the colorimeter when only qualitative or semi-quantitative estimates of the actual enzyme activity were required. Porphyran (or other substrate) solution containing about 20 $\mu$g. of 3:6-anhydrogalactose was mixed with excess of enzyme (0·1–0·2 mg. of freeze-dried extract in 0·1 M-tris–hydrochloric acid buffer, pH 7·6; total volume of digest 0·2–0·4 ml.). After incubation at 35° for the specified period resorcinol–hydrochloric acid reagent (10 ml.) was added and the colour developed as usual.

Blank digests were prepared with both methods by the inclusion of EDTA (0·01 M overall concentration) to inhibit the enzyme.

*Preparation of enzyme.* A single enzyme preparation made as follows was used throughout this work. The entire operation was carried out at 0–4°. Seaweed (Peat & Rees, 1961) was collected at Port Seton, near Edinburgh, on 26 October 1960 (4 kg. wet weight) and extracted with cold sodium carbonate (Rees, 1961*b*). The solution (7 l.; pH 8·3) was adjusted to pH 6·6 with dilute acetic acid. At this stage the extract had measurable activity on porphyran, as when portions (0·2 ml.) were incubated with porphyran (0·17% in 0·1 M-tris, pH 7·6; 0·2 ml.) the reading on the colorimeter increased from 5·4 to 5·5 (15 min.), 5·8 (1 hr.) and 6·1 (4 hr.). Adsorption of the enzyme on calcium phosphate gel was carried out essentially as described previously, except that the process was followed by assaying by the 'semi-quantitative' method the residual anhydrogalactose-synthesizing activity rather than the sulphate-liberating activity. Sufficient gel was added to remove 75% of the enzyme, and after equilibration for 30 min. the calcium phosphate was removed on the centrifuge and washed with sodium acetate (0·1 M, pH 6·5; 2 × 1 vol.; method as previously). Two washings with 0·01 M-potassium dihydrogen phosphate adjusted to pH 7·6 with 3 N-sodium hydroxide (2 × 2 vol.) eluted much inactive material and the gel was then treated with 0·1 M-potassium dihydrogen phosphate, pH 7·6 (2 × 2·5 vol.) and finally with 0·2 M-potassium dihydrogen phosphate, pH 7·6 (1 × 2·5 vol.). These last solutions were bulked (it was not possible to assay since phosphate in this concentration is inhibitory) and adjusted to 0·8 saturation with solid ammonium sulphate. The precipitate was removed on the centrifuge, dissolved in buffer (0·01 M-phosphate, pH 7·6) and dialysed against distilled water at 1° for 36 hr., the water being changed every 12 hr. The resulting solution was freeze-dried to an off-white powder (0·58 g.).

## RESULTS

*Accuracy of the assay method.* When the conditions for colour development were rigidly standardized, the colorimeter readings were reproducible to within 1–2%. Since the colour tends to fade after development, it is desirable, especially when measuring a large number of solutions at the same time, to correct for this. No allowance was made for the contribution to the colorimeter reading of sugars other than 3:6-anhydrogalactose present in the reference polysaccharide. However, Yaphe (1960) has shown that galactose does not seriously interfere with the determination, since it gives less than 1% of the colour given by an equal weight of 3:6-anhydrogalactose.

Although the colorimetric determination of 3:6-anhydrogalactose is highly satisfactory, its application to enzyme assay is less so, since such assay depends upon the measurement of the difference between the amounts of the anhydro sugar present before and after incubation. In experiments where the initial rate of reaction was measured with excess of substrate present, the possible error in measuring the difference was about 10%.

Erroneously high readings on the colorimeter

were obtained when the solution contained oxidizing agents such as nitrate (e.g. the addition of 0·2 m-mole of sodium nitrate to a sample containing 30 $\mu$g. of 3:6-anhydrogalactose gave a reading which was 10% too high), and the use of these was therefore avoided.

*Purified enzyme preparation.* The Rees (1961*b*) method of purification was modified, since it was necessary in the original method to elute the gel many times with 1 M-sodium acetate, the density of which was close to that of the adsorbent, and this meant that prolonged periods of centrifuging were required. The present modification was much quicker, and although it gave a less purified product (tenfold as compared with 22-fold) this might have been due to seasonal variations in the protein constituents of the plant. The yield was 58 % of the original activity. The preparation contained 0·7 % of 3:6-anhydrogalactose.

*Comparison of the amounts of free sulphate liberated and 3:6-anhydrogalactose synthesized.* Porphyran (1 %, w/v; 10 ml.) was mixed with enzyme (0·2 % in 0·04 M-tris buffer, pH 7·6; 10 ml.) and the solution incubated at 35°. Portions were withdrawn at intervals and analysed for free sulphate and total 3:6-anhydrogalactose. The results are shown in Table 1.

Table 1. *Comparison of the amounts of free sulphate liberated and 3:6-anhydrogalactose synthesized*

Values are expressed as $\mu$moles of product/ml. of incubation mixture. For experimental details see text. The incubation mixture contained about 3 $\mu$moles of 3:6-anhydrogalactose/ml. at the start of the experiment.

| Time (min.) | 3:6-Anhydro-galactose | Sulphate |
|---|---|---|
| 0 | 0 | 0 |
| 75 | 1·0 | 1·1 |
| 135 | 1·3 | 1·3 |
| 1020 | 1·6 | 1·5 |

*Activation, inhibition and pH characteristics.* These experiments were performed under the conditions for 'quantitative assay'. The pH–activity curve in 0·01 M-borax–hydrochloric acid buffer coincided within experimental error with that already published for the sulphate-liberating enzyme (Rees, 1961*b*). The results of the activation-inhibition experiments similarly suggested identity with the sulphate-liberating enzyme (Table 2).

*Action on low-molecular-weight potential substrates.* The substances examined were the 'oligosaccharides A and B' derived from porphyran by partial acidic hydrolysis (Peat & Rees, 1961). The incubation conditions were those for 'semi-quantitative assay' described above (24 hr.). No 3:6-anhydrogalactose synthesis was observed with oligosaccharide A [degree of polymerization (D.P.) 5–7], and only slight synthesis (about 5 % of the action on porphyran) with oligosaccharide B (D.P. about 20).

*3:6-Anhydrogalactose synthesis after enzymic depolymerization.* The depolymerase was that already present in the preparations (Peat & Rees, 1961; Rees, 1961*b*). A digest was prepared by mixing crude enzyme (Peat & Rees, 1961; 30 mg.) with water (3 ml.), porphyran (1 %, w/v, solution; 10 ml.), buffer (0·2 M-potassium phosphate, pH 6·5; 3 ml.) and EDTA (0·02 M; 5 ml.). This pH had been found to be optimum for the depolymerase. The function of the EDTA was to inhibit 3:6-anhydrogalactose synthesis. The mixture was divided into two, and one of these portions placed on a boiling-water bath for 5 min. Both portions were then incubated at 35° in the presence of toluene. The specific viscosity of the digest that had not been boiled decreased steadily for 4 days, from 0·34 to 0·06. Both solutions were left in the incubator for a further 5 days, transferred to dialysis sacs, and placed in separate beakers of water (500 ml. at 10–15°) for 4 days. The diffusates

Table 2. *Activation and inhibition of 3:6-anhydrogalactose-synthesizing enzyme*

(Cf. Table 2, Rees, 1961*b*.) Digests were prepared by the 'quantitative assay' method detailed in the Experimental section.

| Reagent | Concn. (mM) | Activation (%) | Inhibition (%) |
|---|---|---|---|
| NaF | 50 | — | 50 |
| KCN | 5 | — | 100 |
| EDTA | 3 | — | 100 |
| EDTA + zinc acetate | 0·5 and 1·5 respectively | — | 0 |
| EDTA + $MgCl_2$ | 0·5 and 1·5 respectively | — | 100 |
| Borax–HCl buffer, pH 7·6 (only electrolyte present) | 10 | 50 | — |
| Sodium phosphate buffer, pH 7·6 (only electrolyte present) | 10 | — | 70 |

were analysed for carbohydrate, with the phenol–sulphuric acid reagents (Dubois, Gilles, Hamilton, Rebers & Smith, 1956), when it was found that only negligible quantities of carbohydrate (< 5%) had passed through the membrane in each case. The solutions were then dialysed exhaustively (running tap water at 10–15° for 3 days), and analysed for combined 3:6-anhydrogalactose and combined sulphate [hydrolysis in N-HCl for 24 hr. followed by spectrophotometric determination of liberated sulphate (Jones & Letham, 1954)]. The solutions were indistinguishable on this basis [(molar ratios for sulphate: anhydro sugar were 1·10:1 (digested) and 1·08:1 (control)]. The action of the enzyme on each was then compared under the conditions for 'semi-quantitative assay' but with 2 mM-zinc acetate present (24 hr. incubation). The 3:6-anhydrogalactose content of the digest containing enzymically depolymerized substrate increased by 41%, whereas that of the control increased by 72%. It is concluded that the 3:6-anhydrogalactose-synthesizing enzyme acts less readily on partially depolymerized porphyran, even when the extent of depolymerization is insufficient to yield dialysable fragments.

*Limit of the enzyme-catalysed reaction.* When mixtures of porphyran and enzyme were incubated until no further synthesis of 3:6-anhydrogalactose occurred, it was invariably found that some ester sulphate remained. This was not unexpected, since there are indications (Turvey & Rees, 1961) that there is more than one type of sulphate ester linkage present in the substrate. When the reaction was allowed to go to completion, the proportion of sulphate ester released varied between about 5% and 50% of the total, it being necessary to add almost an equal weight of enzyme to reach the higher figure. The enzyme undoubtedly became inactivated rather rapidly during incubation. Thus, in one experiment with 5 mg. of enzyme, in 5 ml. of 0·02M-tris buffer, pH 7·6, and 20 mg. of porphyran [this sample was different from that used in all the other experiments, although it was prepared in the same way. It had 12·9% 3:6-anhydrogalactose and 9·8% sulphate (as $SO_3$), and was prepared by Mr T. P. Williams in collaboration with Professor S. Peat and Dr J. R. Turvey. For reasons not yet understood it is not as good a substrate as the initial sample] the proportion of the total ester sulphate that was released with the formation of 3:6-anhydride was 14% in the first 24 hr., and no further synthesis was detected in the following 24 hr. Addition of more enzyme (5 mg.) raised the limit to 22%, and after a third addition the figure was 28%. The absolute limit of enzyme action was established in a separate experiment (7 mg. of porphyran, 5 mg. of enzyme, in 2 ml. of 0·04M-tris buffer), when 49% of the total ester sulphate was released with anhydride formation. This limit could not be passed by the addition of more enzyme (3 mg.)

*Possibility of enzymic modification of porphyran during isolation.* Samples of seaweed were collected at Criccieth, North Wales, on the same day in September. One sample (sample A) was plunged immediately on harvesting into boiling ethanol, and left in the boiling solvent for 5 min. Pigments appeared immediately in the ethanol suggesting that the solvent quickly penetrated the cells. The specimen was then air-dried at 40° for 3 days. Further samples were air-dried at 40° for 3 days (sample B), and air-dried at 40° for 2 days and finally at 110° overnight (sample C). Each was milled, extracted exhaustively with boiling water, and, after clarification of the solutions on the centrifuge, the polysaccharides were isolated by precipitation with ethanol (3–4 vol.). Each sample gave a different yield of polysaccharide (29% from sample A, 22% from sample B, and 21% from sample C), the differences probably being due to removal of different amounts of moisture and other materials from the specimens. The isolated polysaccharide samples were analysed for 3:6-anhydrogalactose by the modified (Peat *et al.* 1961) method of O'Neill (1955). The figures so obtained were within the limits of accuracy of this method (sample A, 8·5; B, 8·4; C, 8·6%). It is concluded that the 3:6-anhydrogalactose-synthesizing enzyme does not act on porphyran to any measurable extent during the normal process of isolation of the polysaccharide.

*Effect of the addition and removal of sulphate.* When sulphate was added to digests prepared for 'semi-quantitative assay', the activity was suppressed, inhibition being complete at 0·2M overall sulphate concentration (added as ammonium sulphate adjusted to pH 7·6 with sodium hydroxide). No reverse reaction could be demonstrated by the addition of further quantities of sulphate. The effect of barium ions on the reaction was also studied (under 'quantitative assay' conditions) to test the possibility that, by removal of the liberated sulphate, the reaction might be driven to a higher limit of conversion than is normally observed. However, it was found that barium ions at a concentration of 10 mM produced 40% inhibition.

## DISCUSSION

The enzymic reaction that is the subject of this paper is detected by an increase in intensity of the Seliwanoff reaction given by the incubation medium. In the light of what is known of the components of porphyran (Peat *et al.* 1961; Turvey & Rees, 1961), and of previous studies of this enzyme system (Rees, 1961*b*), it seems certain

that this is due to the formation of 3:6-anhydro-L-galactose within the polysaccharide from L-galactose 6-sulphate units. Thus the enzymic reaction leading to formation of 3:6-anhydrogalactose is indistinguishable from that leading to liberation of free sulphate from the ester linkages of porphyran in that (*a*) equimolar amounts of Seliwanoff-positive material (calculated as 3:6-anhydrogalactose) and free sulphate are formed, (*b*) the two reactions have similar pH characteristics, (*c*) they are both activated by borate, (*d*) they are both inhibited powerfully by cyanide and metal-binding reagents, and to a certain extent by phosphate, (*e*) the specificities are similar as far as they have been examined, and (*f*) the activity which is abolished by the addition of metal-binding reagents can, in each case, be restored by the addition of zinc but not magnesium.

It is easy to see how de-esterification of the L-galactose 6-sulphate residues which are known to be present could proceed simultaneously with 3:6-anhydro-L-galactose formation, since an analogous chemical reaction is known (Duff & Percival, 1941).

Since 3:6-anhydrogalactose is usually combined in polysaccharides through position 4 and in α-linkage, it is probable that the L-galactose 6-sulphate precursor units are similarly linked. It might be that the failure to achieve removal of more than 50 % of the sulphate is due to the remaining ester's being combined in some other linkage.

It is doubtful whether this enzyme should be called a sulphatase, since it would appear desirable to reserve this term for those enzymes which hydrolyse sulphate esters, i.e. which cleave O–S bonds (Spencer, 1958, 1959), just as phosphatases cleave the O–P bond of phosphate esters. The established mechanism of the alkali-catalysed reaction involves nucleophilic attack on C-6 of the hexose ring by the ionized C-3 hydroxyl group, resulting in fission of the C–O bond of the sulphate ester. If, as seems likely, the enzymic process is similar, then the enzyme is analogous to phosphorylases [acting in reverse and catalysing 'dephosphorolysis' (Baldwin, 1959)] rather than phosphatases. The ester linkage is in each case split at the side nearer the carbon atom and the inorganic anion is released with the simultaneous synthesis of an O–C bond.

It appears that a name such as 'sulphate eliminase' will need to be used if further work supports this preliminary picture. Carbohydrate-metabolizing enzymes that catalyse reactions similar to those effected by alkali are well known, e.g. aldolase, mutarotase (Hough & Jones, 1956). Bacterial hyaluronidase and pectin *trans*-eliminase probably represent other enzymes in this category having polysaccharide substrates (Albersheim, Neukom & Deuel, 1960).

The presence of this enzyme in the seaweed extracts is a strong indication that L-galactose 6-sulphate in the polysaccharide is the immediate biological precursor of 3:6-anhydro-L-galactose. The isolation from *P. perforata* (which is botanically very closely related to, if not identical with, the species used in the present work; E. Conway, personal communication) of uridine diphosphate D-galactose and guanosine diphosphate L-galactose (Su & Hassid, 1960) suggests that these nucleotide sugars are substrates for the synthesis of a DL-galactan which is sulphated, presumably by mechanisms similar to those which occur in the mucopolysaccharide field (e.g. Davidson & Riley, 1960), and some of these sulphate groups are then removed by the mediation of the enzyme, thus introducing 3:6-anhydrogalactose residues. An additional stage which cannot yet be placed in the sequence is partial methylation to give 6-*O*-methyl-D-galactose units.

The enzyme attacks even quite large fragments of porphyran much more slowly than it does the parent polysaccharide. This is in agreement with the rule of Koshland & Stein (1954), the application of which to the present system leads us to expect that since the sugar residue is transferred, with C–O bond fission, the enzyme should exhibit appreciable specificity towards this part of the molecule. It is clear that since prior exposure of porphyran to depolymerase reduces the rate at which 3:6-anhydrogalactose synthesis can proceed, this associated enzyme binders rather than assists synthesis (cf. Peat & Rees, 1961). Indeed the presence of depolymerase in the partially purfied preparation may be one reason (in addition to the instability of the 3:6-anhydrogalactose-synthesizing enzyme) why it is necessary to add large amounts of enzyme preparation in order to achieve high levels of 6-sulphate to 3:6-anhydride conversion, since the substrate is degraded to a molecular size which is suboptimum for 3:6-anhydrogalactose synthesis. It is fortunate, however, that the fragments produced are not small enough to pass through a dialysis membrane, since this simplifies recovery of the enzyme-treated porphyran.

## SUMMARY

1. The liberation of sulphate from the ester linkages of porphyran, which is catalysed by an enzyme preparation from *Porphyra* extracts, is inseparable from the simultaneous synthesis of 3:6-anhydrogalactose units within the polysaccharide.

2. The enzymic reaction is seen to be analogous to the well-known alkaline elimination of sulphate from hexose 6-sulphate derivatives. It is suggested that L-galactose 6-sulphate units in porphyran are

converted, with fission of the C–O bond of the sulphate ester, into 3:6-anhydro-L-galactose residues and that the enzyme is therefore not a true sulphatase.

3. The biosynthesis and structure of porphyran are discussed in the light of the characterization of this enzyme.

4. During the normal processes of collecting, drying and extracting the seaweed, the enzyme does not modify porphyran to any measurable extent.

5. The enzyme shows diminished activity towards the products of partial acidic and enzymic hydrolysis of porphyran, and its action is not reversible.

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# The Inhibitory Effect of 2:4-Dinitrophenol on Metastatic Tissue Calcification Induced by Calciferol

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Whitehead & Weidmann (1959) showed that, in calcifying cartilage in living kittens, the synthesis of adenosine triphosphate (measured by incorporation of $^{32}P$) was inhibited by 2:4-dinitrophenol. It was also found that the uptake of the isotope into bone salt was similarly inhibited only after longer treatment with 2:4-dinitrophenol. Moreover, Gutman & Yii (1949) demonstrated that 2:4-dinitrophenol is capable of inhibiting the calcification of cartilage *in vitro* at a concentration of 0·1 mM.

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Because calcification of the rat aorta, induced by calciferol, is accompanied by a large increase in this vessel's phosphorus content (Gillman, Grant & Hathorn, 1960), we decided to investigate the effect of 2:4-dinitrophenol on mineralization of rat aorta, lung and stomach produced by acute calciferol intoxication. The results clearly indicate that 2:4-dinitrophenol exerts an inhibitory effect on this process.

## METHODS AND MATERIALS

Twenty male Wistar-strain albino rats with a mean weight of 329 ± 7 g. were randomly allocated to each of the following four groups of five rats.

Rats in group 1 were given, twice daily, intraperitoneal injections of 3 ml. of 0·9% sodium chloride solution for

20 days. Group 3 rats were given identical saline injections together with oral calciferol (C. Petersen and Co., Cape Town, South Africa) in daily doses as a solution in arachis oil from day 6 to 10 inclusive, a total of 100 000 units/kg. body wt. being given. Rats in group 2 were given injections, twice daily, of 3 ml. of a saturated solution of 2:4-dinitrophenol in 0·9% sodium chloride for 20 days. Group 4 rats received the same injections as group 2 together with calciferol given orally as in group 3. On day 20 all the animals were killed and the aorta, lungs and stomach dissected out. The tissues were dehydrated with acetone, defatted with ether–alcohol (1:1, v/v) and dried to constant weight at 100°. A second series of animals were treated in the same four groups but were killed and bled from the heart on day 9 of the experiment, i.e. 4 days after starting calciferol treatment; the serum was analysed for calcium and inorganic phosphate.

*Total phosphorus.* Accurately weighed portions of dry fat-free tissue (about 100 mg.) were digested with 1 ml. of a 1:2 (v/v) mixture of perchloric acid (70%) and nitric acid (sp.gr. 1·52) at 150° till clear. The digest and washings were transferred to a 10 ml. volumetric flask and made to volume. Suitable portions (depending on the amount of P present) were transferred to a 25 ml. volumetric flask. To each flask was added 2 ml. of 2N-hydrochloric acid, followed by 2 ml. of reducing-agent solution (Metol 1%; sodium sulphite 5%) and 2 ml. of ammonium molybdate solution (2·5%). The volume of the solution was adjusted to 25 ml. with water and mixed thoroughly. A phosphate standard (= 1 mg. of P) was treated in the same way. After 10 min. the solutions were transferred to 1 cm. cuvettes and the extinctions determined at 660 mμ.

*Calcium determinations.* Portions of the digest were titrated with 0·01N-ethylenediaminetetra-acetic acid with the calcium indicator of Diehl & Ellingboe (1956) screened with thymol phthalein (Tucker, 1957) in the presence of 1 ml. of 2·5N-sodium hydroxide. Serum calcium was determined similarly in volumes of 1 ml.

*Serum inorganic phosphate.* This was determined by a modification of the method of Fiske & Subbarow (1925).

*Statistical methods.* Except for serum calcium and serum inorganic phosphate, where the variances were homogeneous, analysis of variance was performed on the results after logarithmic transformation in order to render the variances homogeneous (Maxwell, 1958). In order to assess the significance of differences between group means, 2-tailed *t* tests were used at the 5% level of significance, the standard error of the difference having been calculated from the residual error variance in the analysis of variance (Maxwell, 1958). In Table 1, significant differences in means are indicated by the sign '>' whereas groups not differing significantly from each other are separated by a comma.

## RESULTS

The mean percentage calcium and phosphorus contents of the tissues, together with the mean aortic dry weights and the serum calcium and inorganic phosphorus values, are given in Table 1. This also includes an analysis of variance of the individual results obtained in 20 animals.

The present results confirm our previous observation (Gillman *et al.* 1960) that, with calciferol alone, treatment resulted in an approximately 2½-fold increase in the mean aortic dry weight; for this reason the results for calcium and phosphorus are expressed in mg./aorta. Table 1 also shows that 2:4-dinitrophenol administered during calciferol treatment significantly reduced the effect of calciferol on total aortic dry weight, although 2:4-dinitrophenol alone had no effect at all on the aortic weight.

Both the calcium and phosphorus contents of the aorta were greatly increased by calciferol treatment; 2:4-dinitrophenol alone had no effect on these constituents, but, when given simultaneously with calciferol, significantly reduced, by approximately one-half, the degree of mineralization induced by calciferol alone. The calcium:phosphorus ratios of the aortae also tended to be reduced in the two groups receiving 2:4-dinitrophenol injections, but, owing to wide variations in individual values, the differences were not significant.

Similar results were obtained for lung and stomach; calciferol produced significant increases in the calcium and phosphorus contents of these organs and 2:4-dinitrophenol given simultaneously reduced this effect.

The results of the serum calcium and inorganic phosphate determinations showed that calciferol markedly increased both these serum constituents. However, despite its marked effects on tissue mineralization, 2:4-dinitrophenol given simultaneously with calciferol did not affect the increases in the serum calcium and inorganic phosphorus. It would thus appear that the inhibitory effect of 2:4-dinitrophenol on calcification is not mediated via any overt action on blood calcium or phosphate levels.

In Table 2 the results for tissue calcium and total phosphorus are expressed in the form of increments over the saline control values. The calcium:phosphorus ratios obtained, whether calciferol was given alone or with 2:4-dinitrophenol, tended to be in the region of 2:1. This indicates that the mineral deposited is calcium phosphate with a calcium and phosphorus composition similar to that of bone salt. Table 2 also shows the extent to which 2:4-dinitrophenol inhibited the calciferol-induced metastatic mineralization. This Table reveals that for the tissues other than aorta, treatment with 2:4-dinitrophenol given together with calciferol resulted in an equal reduction of both calcium and phosphorus as compared with the amounts of these constituents found in rats treated with calciferol only. In the aorta, however, the degree of inhibition of the calciferol-induced calcium increase due to 2:4-dinitrophenol was 20% greater than its effect on phosphorus accumulation. In consequence the Ca:P ratio of the aortae of rats in

Table 1. *Effects of calciferol and 2:4-dinitrophenol, given separately and together, on the calcium and phosphorus contents of rat aorta, lung and stomach*

NS, Not significant.

| | | Serum | | | Aorta | | | |
|---|---|---|---|---|---|---|---|---|
| Group | Treatment | Ca (mg./100 ml.) | Inorganic phosphate (mg./100 ml.) | Ca–P product | Wt. (mg.) | Ca (mg./aorta) | P (mg./aorta) | Ca/P |
| 1 | Saline | 11·9±0·2* | 6·5±0·2 | 77 | 16·7±1·2 | 0·206±0·064 | 0·073±0·006 | 2·84 |
| 2 | DNP | 11·6±0·2 | 6·6±0·6 | 77 | 16·7±1·0 | 0·108±0·014 | 0·079±0·011 | 1·37 |
| 3 | Calciferol + saline | 14·2±0·3 | 8·8±0·5 | 125 | 43·8±5·5 | 6·23 ±0·91 | 2·30 ±0·33 | 2·71 |
| 4 | Calciferol + DNP | 15·2±0·1 | 8·4±0·2 | 128 | 28·1±4·0 | 2·88 ±1·03 | 1·15 ±0·31 | 2·51 |

Analysis of variance

| | Serum | | | | Aorta | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *F*† | *P* | *F* | *P* | *F* | *P* | *F* | *P* | *F* | *P* |
| Main effects: | | | | | | | | | | |
| Calciferol | 98·0 | — | 18·86 | <0·001 | 42·57 | — | 44·16 | <0·001 | 119·53 | <0·001 |
| DNP | 2·06 | — | 0·11 | NS | 4·25 | — | 4·56 | <0·05 | 3·14 | NS |
| Interaction: | | | | | | | | | | |
| Calciferol – DNP | 6·04 | <0·05 | 0·50 | NS | 4·60 | <0·05 | 1·18 | NS | 4·06 | NS |
| Significant differences between means‡ | 4 > 3 > 1, 2 | | 3, 4 > 1, 2 | | 3 > 4 > 1, 2 | | 3 > 4 > 1, 2 | | 3 > 4 > 1, 2 | |

| | | Lung | | | Stomach | | |
|---|---|---|---|---|---|---|---|
| Group | Treatment | Ca (% dry wt.) | P (% dry wt.) | Ca/P | Ca (% dry wt.) | P (% dry wt.) | Ca/P |
| 1 | Saline | 0·17±0·03 | 0·84±0·01 | 0·20 | 0·12±0·01 | 0·89±0·03 | 0·13 |
| 2 | DNP | 0·11±0·01 | 0·83±0·02 | 0·13 | 0·15±0·02 | 0·85±0·01 | 0·18 |
| 3 | Calciferol + saline | 1·10±0·41 | 1·38±0·20 | 0·80 | 0·95±0·33 | 1·23±0·16 | 0·77 |
| 4 | Calciferol + DNP | 0·33±0·09 | 0·93±0·04 | 0·35 | 0·46±0·09 | 1·06±0·06 | 0·43 |

Analysis of variance

| | Lung | | | | Stomach | | | |
|---|---|---|---|---|---|---|---|---|
| | *F* | *P* | *F* | *P* | *F* | *P* | *F* | *P* |
| Main effects: | | | | | | | | |
| Calciferol | 31·44 | <0·001 | 17·54 | — | 44·72 | <0·001 | 13·15 | <0·01 |
| DNP | 9·53 | <0·01 | 7·50 | — | 1·04 | NS | 1·74 | NS |
| Interaction: | | | | | | | | |
| Calciferol – DNP | 1·82 | NS | 6·22 | <0·05 | 3·23 | NS | 0·43 | NS |
| Significant differences between means‡ | 3 > 4, 3 > 1, 2 4 > 2 | | 3 > 4, 1, 2 | | 3 > 4, 1, 2 | | 3 > 1 4 > 2 | |

* Mean ± S.E. [S.E. calculated as S.D./$(n-1)$].
† Ratio of the variance due to the effect considered to the error variance (tested with 1 and 16 degrees of freedom).
‡ Standard error of difference of means calculated from error variance; 2-tailed *t* test with 5% level of significance.

Table 2. *Increases in tissue concentrations of calcium and phosphorus induced by calciferol given alone and with 2:4-dinitrophenol*

| | Calciferol alone Increments* | | | Calciferol + 2:4-dinitrophenol Increments | | | Inhibition of calciferol effect (%) | |
|---|---|---|---|---|---|---|---|---|
| | Ca | P | Ca:P | Ca | P | Ca:P | Ca | P |
| Aorta | 12·8 | 4·8 | 2·7:1 | 7·3 | 3·2 | 2·3:1 | 43 | 33 |
| Lung | 0·93 | 0·54 | 1·7:1 | 0·16 | 0·09 | 1·8:1 | 83 | 83 |
| Stomach | 0·83 | 0·34 | 2·5:1 | 0·34 | 0·15 | 2·3:1 | 59 | 56 |

* Experimental level – saline control level (expressed as percentage of dry fat-free tissue).

group 3 was significantly greater than of those in group 4. Although the reasons underlying this differential effect of 2:4-dinitrophenol on the calcium and phosphorus levels in the aortae of calciferol-intoxicated rats remains obscure, nevertheless this finding indicates differences in the responses of the various tissues to the action of 2:4-dinitrophenol.

## DISCUSSION

In earlier studies by Trout (1958) and by Gillman *et al.* (1960) it was shown that mineral continued to accumulate in the aortae of calciferol-intoxicated rats long after administration of this compound was stopped and after the serum calcium level had returned to normal. It therefore appears that, once initiated, calcification of the aorta, under these conditions, follows an irreversible course, since even 160 days after calciferol intoxication there was no evidence of a reduction in the mineral content of such injured aortae. Aorta appears to differ in this respect from other tissues, since in the heart, for instance, the calcium disappeared within a relatively short time. The mechanism underlying these differences in reactivity is at present obscure.

Since the increases in blood calcium and inorganic phosphorus, induced by calciferol, were not affected by simultaneous administration of 2:4-dinitrophenol, it would appear that 2:4-dinitrophenol exerts its action directly on the tissues. In this respect our results may perhaps be better understood on the basis of the finding of Whitehead & Weidmann (1959) for kitten cartilage in which 2:4-dinitrophenol was found to inhibit synthesis of adenosine triphosphate and reduce the uptake of $^{32}P$ into the bone salt. Cartier & Picard (1955) suggested that adenosine triphosphate is responsible for the initiation of calcification by the formation of seed crystals, and the results of Whitehead & Weidmann (1959) are in agreement with this concept. Our own finding, that 2:4-dinitrophenol depresses the degree of soft-tissue mineralization induced by calciferol, is also in conformity with those of the above authors. This similarity in response to 2:4-dinitrophenol of calcification processes in cartilage, lung, stomach and aorta may indicate that all these tissues calcify by a common mechanism involving in part perhaps adenosine triphosphate and adenosine triphosphatase balance. A further indication of this possibility emerges from the similarity in Ca:P ratio of the mineral salts deposited in all the above tissues (see Table 2).

A reduction in the adenosine triphosphate content of the tissues could also result from a stimulation of adenosine-triphosphatase activity, especially as Chappell & Perry (1954) showed that skeletal muscle adenosine triphosphatase is stimulated by 2:4-dinitrophenol. Stimulation of chick-embryo mitochondrial adenosine triphosphatase by 2:4-dinitrophenol has also been reported by Carey & Greville (1959) and the activation of three rat-liver adenosine triphosphatases by 2:4-dinitrophenol was shown by Myers & Slater (1957). Further evidence for this action of 2:4-dinitrophenol has recently been given by Penniall (1960). Wakid (1960) has also shown that in the absence of $Mg^{2+}$ ions the adenosine-triphosphatase activity of rat myometrial mitochondria was increased about four times by 0·1 mM-2:4-dinitrophenol. $Mg^{2+}$ ions alone produced a tenfold to 20-fold increase in the adenosine-triphosphatase activity. This last observation is of interest in view of Selye's (1958) finding that administration of magnesium chloride prevented calcification of the aortae of rats intoxicated with both dihydrotachysterol and sodium phosphate. This effect of $Mg^{2+}$ ions on the action of drugs studied by Selye may perhaps have been due to the increased adenosine-triphosphatase activity induced by $Mg^{2+}$ ions.

However, the level of tissue adenosine triphosphate does not appear to be the only factor involved in calcification under the action of calciferol since skeletal muscle, which is relatively rich in adenosine triphosphate, calcifies only to a small extent compared with aorta.

## SUMMARY

1. 2:4-Dinitrophenol has been found to exert an inhibitory effect on soft-tissue calcification induced by calciferol.

2. 2:4-Dinitrophenol did not affect the increases in blood calcium and inorganic phosphate induced by calciferol and appears to act directly on the tissues.

3. It is suggested that the above effect of 2:4-dinitrophenol on calcification may be in part at least due to its known effects on adenosine triphosphate synthesis and adenosine-triphosphatase activity.

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# Structural Changes in Collagen

## 2. ENHANCED SOLUBILITY OF BOVINE COLLAGEN: REACTIONS WITH HYDROGEN PEROXIDE AND SOME PROPERTIES OF SOLUBLE EUCOLLAGEN*

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Evidence by Fitton-Jackson (1953) and others shows that the generation of collagen *in vivo* takes place intercellularly to the stage of a soluble precursor and becomes insoluble extracellularly by some type of aging process likely to involve cross-linking. A substantial fraction of embryonic bovine collagen is soluble in citrate buffer (Gebhardt, 1960) and accounts for more than 13% of the total collagen at birth. The small fraction of adult human or bovine collagen which is solubilized in citrate buffer generally represents less than 1% of the total (Plotnikova, 1947, 1949) or about 4% in calf skin (Bowes, Elliott & Moss, 1955). The fraction similarly dispersed from the tail tendon of the rat or from teleostean collagen is considerably greater. Dumitru & Garrett (1957) obtained nearly quantitative solution of rat-tail tendon in distilled water at pH 6 after treatment with 0·5 M-sodium dihydrogen phosphate, whereas bovine tendon did not dissolve in this way. Clearly, these highly dispersible collagens are different from bovine or human collagen. One common feature of acid-soluble collagens from a wide variety of connective tissues and species appears to be the retention of the highly ordered collagen structure as indicated by well-orientated X-ray-diffraction patterns and typical collagen 640 Å spacings, confirmed by electron microscopy (Cowan, North & Randall, 1955; Randall, Booth, Burge, Jackson & Kelly, 1955; Schmitt, Gross & Highberger, 1955) and by high intrinsic viscosity in dilute solution (Boedtker & Doty, 1956; Gallop, 1955; Doty & Nishihara, 1958). Methods that lead to extensive solubilization of bovine collagen, such as autoclaving in water above 100°, heating in ethylenediamine (Solomons, 1956) or potassium phosphate (Bate-Smith, 1938), also result in loss of collagen structure.

Courts (1960) has described the conversion of collagen into eucollagen in which substantial disorganization of covalently bonded structure is likely, but regions of hydrogen bonding maintain a stable collagen-type structure. The present study suggests that, during the extensive dispersion of eucollagen into acid buffer solutions, the retention of the collagen fold is more likely than conversion into gelatin; the material thus obtained has therefore been termed 'soluble eucollagen'. It seems probable that the conversion of collagen into eucollagen is a chemical reversal of the aging process. The reactions described in the present work have made possible a large-scale study of a solubilized collagen from a single batch of bovine connective tissue. The method has an added advantage of freeing the tissue from much of the non-collagen impurity. The present account of the fibre-forming properties is a more detailed study of this new form of collagen than that published by Courts & Stainsby (1959). The X-ray diffraction and electron-microscopy work on the fibres reconstituted from soluble eucollagen is reported elsewhere (Little, Kelly & Courts, 1961).

The alternative available method of non-thermal solubilization of native bovine collagen is by the action of lyotropic agents (Gustavson, 1955). Because of the extensive breaking of hydrogen bonds this type of reaction invariably gives gelatin (Courts, 1958), and the rigid rod-like molecular structure associated with native collagen (Boedtker

* Part 1: Courts (1960).

& Doty, 1956) is no longer discernible. The molecular weight of the gelatins, however, may still be substantial.

Recent interest in the function of polysaccharides in the collagen environment has promoted studies on the reaction of collagen with sodium periodate (Grassmann & Kuhn, 1955; Hörmann & Fries, 1958), and with hydroxylamine (Gallop, Seifter & Meilman, 1959). Gustavson (1957) describes the lyotrophic effects of sodium periodate and perchlorate on collagen. It would appear that no previous study has been made with hydrogen peroxide on intact collagen. With gelatin at 40°, 1·7 M-hydrogen peroxide has the interesting property of impairing the viscosity without significant effect on the rigidity factor (P. R. Saunders, personal communication). In view of the implication in this observation that hydrogen peroxide can catalyse controlled peptide-bond hydrolysis, some investigations have been made on the reaction of collagen with 1·7 M-hydrogen peroxide, from which it is clear that the collagen will disperse with transformation into gelatin, provided that a mechanism that will break hydrogen bonds acts concomitantly.

In the absence of any known enzymic reaction facilitating the dispersion of uterine collagen after parturition (Brown, Consden & Glynn, 1958) it is possible that a catalysed hydrogen-bond-breaking mechanism may be involved. The present work on bone collagen is interim to a study on uterine collagen.

## MATERIALS AND METHODS

*Collagen.* The material used was the ox-bone collagen described by Courts (1960).

*Soluble eucollagen.* Collagen was converted into eucollagen by the procedure of Courts (1960), by the action of NaOH in the presence of $Na_2SO_4$ for 10 days at 20°. The eucollagen was freed from fluid by straining, washed in running water for 30 min. and then allowed to go through isoelectric shrinkage (as indicated by the translucent pieces' turning opaque) by suspension in 0·02 M-acetic acid for 4 hr. Shrinking occurs in the range pH 4–4·3. The pieces were then washed in running water for 1 day. The swollen eucollagen (50 g.) was removed on a nylon mesh and kept in one of the buffer solutions (1 l.) described below for 1 day at 20° with occasional stirring.

*Buffer solutions.* The addition of 0·05 mole of sodium citrate/l. to citric acid (0·1 M; pH 2·2) gave the pH 3·7 buffer used by Gallop (1955) and Boedtker & Doty (1956). Acetate buffer (pH 3·6) was obtained by adding 5 ml. of N-NaOH to 1 l. of 0·15 N-acetate acid. Lactate buffers in the range pH 2·2–3·8 were prepared by adding N-NaOH to 0·15 N-lactic acid. Mandelic acid (0·15 N) was used at pH 3·2.

*Hydrogen peroxide.* AnalaR-grade (British Drug Houses Ltd.) 30% (w/v) (8·6 M) was diluted to the indicated strengths.

*Mucoprotein.* A crude preparation of osseomucoid was made by the propan-2-ol fractionation of an acid-processed ossein gelatin. This procedure concentrated the bulk of the mucoid into fraction 6 (Courts, 1959).

*Amino acid analysis.* Protein hydrolysates of dried, low-ash-content materials were assayed for amino acid composition by the Moore & Stein (1951) method as described by Eastoe (1955). The experiments were carried out by Dr A. A. Leach, Miss J. E. Long and Mr A. P. Williams.

*N-Terminal amino acid residues.* The method of Sanger (1945) was used. The procedure for gelatin was as described by Courts (1954) and for collagens by Courts (1960).

(*a*) Reaction of soluble eucollagen with fluorodinitrobenzene. Soluble eucollagen (0·5 g.) in citrate buffer was adjusted to pH 8 with N-NaOH and shaken with 1-fluoro-2:4-dinitrobenzene (1·4 g.) at 20° for 1 day. The yellow dinitrophenyl derivative separated out. This was suspended in water, acidified with 0·01 N-HCl, washed in water and dried *in vacuo* over $P_2O_5$. The dried derivative was then washed in several changes of acetone until no further yellow colour was discharged.

(*b*) Hydrolysis. Dinitrophenyl-protein (500 mg.) was heated with 5·5 N-HCl (10 ml.) for 16 hr. in sealed tubes at 100°. The subsequent extraction of dinitrophenyl-amino acids and their identification and quantitative determinations closely followed the procedure of Courts (1954).

*Dialysis.* Solutions were dialysed in cellulose tubing against running tap water. The complete precipitation of fibres from acid-soluble eucollagen required 3 days. The complete removal of hydrogen-bond-breaking reagents from gelatin solutions was more prolonged, but in 1–2 days (depending upon the protein concentration) a gel formed, which was purified by washing in running water for 1 day. With collagen from dura mater, Brown *et al.* (1958) recommend 10 days at 37° in 0·9% $NaHCO_3$ to remove sodium salicylate completely.

Washing is preferably carried out on strips of gel, 2 in. × ½ in. × ⅛ in. approx. The removal of $H_2O_2$ from gels was usually achieved in a few hours in this way and was probably facilitated by the hypochlorites present in tap water.

*Soluble eucollagen concentration.* (*a*) Where sufficient quantities were available, the most convenient method was to dialyse about 100 ml. of solution and centrifuge the fibre suspension at 8000*g* for 30 min. This gave a fibre mass containing 3–5% of protein, which was dried *in vacuo* over $P_2O_5$ and weighed. (*b*) Alternatively the hydroxyproline content of the protein solution was determined by the method of Neuman & Logan (1950) as modified by Leach (1960). In view of the hydroxyproline content of soluble eucollagen given in Table 1, the factor taken for converting hydroxyproline into collagen content was 100/14·9. Methods (*a*) and (*b*) were in good agreement.

*Isoionic* pH (p*I*). Gelatin was deionized by passing through mixed-bed resins (Janus, Kenchington & Ward, 1951), and the pH of the emerging solution measured.

*Measurements of* pH. Measurements were made in the Cambridge pH meter with a glass electrode and a saturated calomel electrode (Cambridge Instrument Co. Ltd., London).

*Melting and shrinkage of soluble eucollagen.* The measurement of shrinkage temperature ($T_s$) of eucollagen is described by Courts (1960). For reconstituted soluble eucollagen the dialysed solids were deposited by centri-

fuging at 8000*g* to increase the protein proportion to about 4%. A small quantity (about 1 g.) was smeared as a thin film around the inside wall of a test tube by using a glass rod. The test tube was clamped in a boiling tube containing water so that the level of water appeared just above the film. The water temperature was then raised through 1°/min. The melting of the soluble eucollagen fibres, as indicated by the film's collapsing, was readily observed.

*Precipitation of soluble eucollagen fibres*

*Dialysis.* If the initial concentration of eucollagen in solution is greater than 0·05%, the precipitate is likely to be gelatinous in appearance.

*Salting out.* Solutions of soluble eucollagen at pH 2·2, 3·7 and 5·0 were treated with M-NaCl, M-$Na_2SO_4$, chondroitin sulphate (L. Light and Co. Ltd.) and mucoprotein preparation.

*Breaking of hydrogen bonds.* In experiments on the conversion of soluble eucollagen into gelatin at 20° with hydrogen-bond-breaking reagents (Courts, 1958), it was observed that small quantities of these reagents induced fibre formation. Several 10 ml. portions of soluble eucollagen (0·1%, w/v) at pH 2·2 were treated with 0·1–1·5 ml. of (1) 4 M-sodium salicylate, (2) 4 M-KSCN, with stirring and the mixtures allowed to equilibrate for 1 day. The turbidity developed was measured at 540 mμ (EEL portable colorimeter; green filter) and the pH of each system was measured.

*Turbidity–pH relationship.* Soluble eucollagen in acid solution shows turbidity when adjusted to pH 4–5. This is a reversible reaction and shows a maximum turbidity effect. A similar behaviour is shown by gelatin in water, in which instance the pH of maximum turbidity indicates the isoelectric point of the gelatin (Briefer, 1929). With salt-free material this is also the isoionic point (Janus *et al.* 1951).

A solution of soluble eucollagen (0·1%, w/v) was divided into several 10 ml. portions, each of which was adjusted with N-NaOH to a pH between 4 and 5. The precipitates were allowed to equilibrate for 1 day at 20°, after which turbidity and pH values were measured. Wood & Keech (1960), in experiments on the precipitation of acetic acid-soluble calf-skin collagen, found that 1·5 hr. was required for equilibrium at 25°, with similar effects at 0°. However, the partial reversal, which these authors found after 16 hr. at 0°, indicates that this may be the time required for true equilibrium. The emphasis which Wood & Keech (1960) give to a strict experimental routine in collagen precipitation work is confirmed in the present work.

The effect of varying the molarity of buffer salts, and any specific acid radical effect due to acetate, citrate, lactate, mandelate and chloride, has been examined with solutions of eucollagen prepared by the following methods: (1) a single batch of eucollagen was divided for treatment with different buffer systems or with buffers at different concentrations; (2) soluble eucollagen in 0·15 M-citrate was dialysed against citrate buffers of lower molar concentrations; (3) soluble eucollagen, obtained by extraction in 0·1 M-citric acid, was dialysed against tap water, and the dialysis residue redispersed in buffers of various concentrations.

A similar control experiment was carried out with a gelatin (0·3%, w/v) of p*I* 4·9 in 0·1 M-citrate buffers over the range pH 3–6.

*Solubilization of collagen with hydrogen peroxide*

*At 21° in the presence of reagents breaking hydrogen bonds.* Collagen (100 g.) was treated with the reagent (500 ml.) and 30% $H_2O_2$ (125 ml.; final concn. 1·7 M). The reagents used were 4 M-sodium salicylate, 4 M-LiBr, 8 M-$CaCl_2$, expressed as final concentrations. The same reagents (625 ml.) were also used to treat the collagen in the absence of $H_2O_2$. In addition the effect of 1·7 M-$H_2O_2$ alone was examined. (The addition of $H_2O_2$ to KSCN produced a delayed but violent reaction in which S was deposited. No experiments were made therefore with this reagent.)

Experiments were carried out in closed jars at 21° for 4 weeks with occasional stirring. After straining off the solids on to nylon mesh, the solutions were filtered and dialysed.

*Treatment at 60°.* Collagen (100 g.) was allowed to equilibrate overnight in 1·7 M-$H_2O_2$ (500 ml.) and the mixture heated at 60° for 2½, 3 and 3½ hr. The solutions were quickly cooled to form gels, which were cut into strips and washed in running water. The residues were washed free from $H_2O_2$ and either heated in water at 60° or immersed in 4 M-sodium salicylate for 1 day at 18° and dialysed. Further gels were obtained in this way.

*Effect of sodium sulphate on the peroxide reaction.* The stability of the collagen–$Na_2SO_4$ system against hydrolysis by 0·14 N-$H_2SO_4$ at 80° has been demonstrated by Deutches Gelatine Fabriken (1952) and some chemical aspects of the reaction described by Courts (1960). The effect of $Na_2SO_4$ on the conversion of collagen into gelatin at 60° by 1·7 M-$H_2O_2$ was examined by soaking the collagen in 20% $Na_2SO_4$ overnight, then adding 30% (w/v) $H_2O_2$ to the stated strength. The mixture was then heated at 60° for (1) 3½ hr., and (2) 4½ hr. The solutions were dialysed.

*Effects of peroxide on certain amino acids.* Potential alterations in the structure of certain amino acids, which are constituents of collagen and gelatin, were separately investigated by heating them in 1·7 M-$H_2O_2$ at 60° for 3½ hr. After evaporating to dryness *in vacuo*, the residue was taken up in water and examined along with controls by paper chromatography, by the descending method with butanol–acetic acid–water (4:1:5, by vol.). Serine, aspartic acid, proline and hydroxyproline, methionine, histidine and also glycylglycine were treated in this way.

*Catalytic effect of peroxide in gel liquefaction.* Sodium bromide and urea are listed by Courts (1958) as 'weakly active' in their relative efficiency in liquefying a gelatin gel. These reagents have now been re-examined in the presence of $H_2O_2$. Gelatin granules (0·5 g.) of $600 \pm 100\,\mu$ in a stoppered test tube were allowed to swell in water (5 ml.) for 30 min. at 18°. The reagent (5 ml.) at twice the indicated strength was added and the swollen mass agitated with a glass rod for a few seconds. The time taken for the gelatin gel to liquefy completely was measured.

*Gel strength.* This was measured at 10° on gelatin gels (5·65%, w/v) which had matured at 10° for 18 hr. according to British Standards Institution (1944) 757. A Bloom gelometer (Griffin and George Ltd., London) was used and the results were expressed in 'grams Bloom'.

*Viscosity.* The viscosity of gelatin was measured on sols at 5·65% (w/v) at 40° in an Ostwald-type viscometer, no. 2 [British Standards Institution (1937) 188].

## RESULTS

### *Soluble eucollagen*

*Amino acid composition.* The complete amino acid assay for a citric acid-soluble eucollagen preparation is given in Table 1 and compared with a typical analysis given by Eastoe (1955) for a gelatin obtained from ox bone by an alkaline pretreatment. The results represent a single analysis except for hydroxyproline, which was carried out in quadruplicate. Probable errors for the analysis are as given by Eastoe (1955). Hydroxyproline values were reproduced within 2% of the mean. In general, the results are in close agreement with analyses for bovine collagens and gelatins (Eastoe, 1955; Eastoe & Leach, 1958). Two significant differences are discussed below.

N-*Terminal amino acid analysis.* Values obtained for citrate-soluble eucollagen in residues/100 kg. of protein gave a total of 1·62 *N*-terminal amino acid residues. Assuming one *N*-terminal residue for each protein chain, this total is equivalent to a number-average chain weight ($C_n$) of 62 000 (defined in Courts & Stainsby, 1958). Similar values were obtained for the eucollagen ($C_n$, 62 000) before citrate-buffer treatment, and for the gelatin ($C_n$, 60 000) derived from the same eucollagen sample by hydrothermal shrinkage at 50°.

*Melting and shrinkage temperatures.* With a reconstituted form of soluble eucollagen, pH 6, the smeared film had an opaque appearance. On raising the temperature the opacity was retained. The fibres melted sharply at 43° to form an opaque liquid. A sharp-melting phenomenon is contrary to usual observations on collagen or eucollagen. The melt did not set to a gel on cooling, indicating that, at this stage, transition to gelatin had not occurred. This was confirmed by redispersing (1 g.) in 0·1 M-citric acid and measuring the viscosity at 20°. The unheated fibres were similarly redispersed and measured as a control. The 43° melt gave a relative viscosity ($\eta_{rel.}$) of 8·8 approximating to the control $\eta_{rel.}$ 7·5. Both of these dispersions, when heated to 44°, gave $\eta_{rel.}$ 1·2, measured at 20°. The results clearly show the 43° melt to have retained a collagen-type structure.

On raising the melt to 44° or above, it clarified and set to a gel on cooling. As a result of the transition to gelatin the viscosity in 0·1 M-citric acid was considerably reduced ($\eta_{rel.}$ 1·2).

*The isoionic* pH. The mixed-bed deionization technique of Janus *et al.* (1951) is unsuitable for determining the isoionic pH (p*I*) of a protein solution, such as soluble eucollagen, which relies upon the environmental ions for solvation. It is reasonable to assume, however, that the transformation of soluble eucollagen into gelatin at neutrality would not involve alterations to charged groups. The derived gelatin was deionized and found to be isoionic at pH 4·8, a value in accord with the low amide-nitrogen value for soluble eucollagen given in Table 1. A similar procedure was adopted by Jackson & Neuberger (1957) for determining the p*I* of rabbit-skin soluble collagen.

Table 1. *Amino acid composition and* p*I of soluble eucollagen and peroxide precursor gelatin*

Values are given as g. of amino acid/100 g. of dry ash-free protein.

| | Soluble eucollagen | $H_2O_2$ extracted | Bone gelatin (Eastoe, 1955) |
|---|---|---|---|
| Alanine | 11·6 | 10·9 | 11·3 |
| Glycine | 26·1 | 26·7 | 27·2 |
| Valine | 2·4 | 2·5 | 2·7 |
| Leucine | 3·4 | 3·5 | 3·4 |
| Isoleucine | 1·4 | 1·4 | 1·5 |
| Proline | 16·3 | 15·0 | 15·5 |
| Phenylalanine | 2·2 | 2·3 | 2·4 |
| Tyrosine | 0·15 | 0·5 | 0·2 |
| Serine | 3·9 | 5·0 | 3·7 |
| Threonine | 2·3 | 3·0 | 2·3 |
| Methionine | 0·6 | 0·2 | 0·6 |
| Arginine | 8·6 | 9·1 | 9·0 |
| Histidine | 0·5 | 0·4 | 0·70 |
| Lysine | 4·7 | 4·0 | 4·3 |
| Aspartic acid | 6·3 | 7·1 | 6·7 |
| Glutamic acid | 10·8 | 11·8 | 11·6 |
| Hydroxyproline | 14·9 | 13·8 | 13·3 |
| Hydroxylysine | 0·8 | 0·9 | 0·7 |
| Total | 116·9 | 118·1 | 117·6 |
| Amide N | 0·10 | 0·6 | 0·24 |
| p*I* | 4·8 | 5·7 | 4·9 |

### *Turbidity studies*

*Relationship with* pH. The development of turbidity in dilute solutions of gelatin in water is a function of pH and is maximal at the isoionic pH (Briefer, 1929; Janus *et al.* 1951). A similar turbidity–pH relationship has been noted with soluble eucollagen dispersed in certain buffer solutions and over a range of buffer concentrations. The results showing the pH of maximum turbidity are summarized in Table 2.

*Citrate.* The development of turbidity in soluble eucollagen–citrate is confined to the range pH 4–4·6 for 0·15 M-buffer, with a well-defined maximum at pH 4·34. This is illustrated in curve *A*, Fig. 1. Of the solvents examined here, citrate is unique in showing a single narrow peak. In addition, curves prepared from results with 0·05 M-, 0·075 M- and 0·125 M-citrate were indistinguishable from curve *A*, Fig. 1. The alkali-processed gelatin in 0·1 M-citrate showed no turbidity from pH 3 to 6. Thus the citrate has inhibited the isoelectric behaviour in gelatin.

*Acetate.* Soluble eucollagen in 0·15 M-acetate gave the broader turbidity–pH range of the type

shown in curve *B*, Fig. 1. Although a reasonably well-defined maximum occurred at pH 4·4, secondary influences are evident about pH 4·8.

*Mandelate.* Where 0·15M-mandelic acid was the solvent for soluble eucollagen, the curve resembled that for acetate.

*Lactate.* The results were similar to curve *B*, Fig. 1. The turbidity maximum was reasonably defined at pH 4·3 ± 0·1 where the lactate concentration was 0·15M or 0·1M. Below this concentration the turbidity maximum was more difficult to define.

Table 2. *Summary of results for turbidity maxima*

| Solvent | Molarity | Method of solution | pH of max. turbidity |
|---|---|---|---|
| Citrate | 0·05 | 1 | 4·30 |
| | 0·075 | 1 | 4·30 |
| | 0·10 | 1 | 4·35 |
| | 0·125 | 1 | 4·33 |
| | 0·15 | 1 | 4·36, 4·32, 4·34 |
| | 0·04 | 2 | 4·22 |
| | 0·06 | 2 | 4·36 |
| Mandelate | 0·15 | 1 | 4·36 |
| HCl | 0·01 | 1 | 4·38 |
| Acetate | 0·15 | 1 | 4·35 |
| Lactate | 0·05 | 3 | 4·25–4·45 |
| | 0·085 | 3 | 4·3–4·5 |
| | 0·10 | 3 | 4·33 |
| | 0·15 | 3 | 4·4 (approx.) |

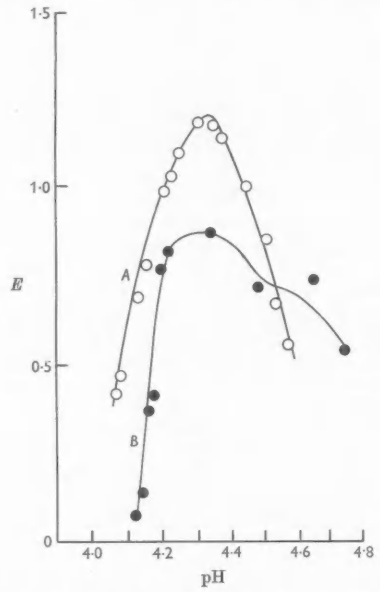


Fig. 1. Turbidity change with pH of soluble eucollagen. Curve *A* represents the changes obtained in 0·05M-, 0·075M-, 0·125M- and 0·15M-citrate buffers. Curve *B* represents the changes obtained in 0·15M-mandelic acid. Changes of systems in 0·2M-acetate, 0·15M-lactate buffers and in 0·01N-HCl (0·05M-NaCl present) were similar to curve *B*.

*Hydrochloric acid–sodium chloride.* In this experiment soluble eucollagen had been extracted in 0·01N-HCl and NaCl equivalent to 0·05M added to facilitate pH adjustment. The turbidity–pH relationship was again similar to curve *B*, Fig. 1, in which the maximum at pH 4·38 resembled the curves obtained with eucollagen in organic acids. The secondary effects, however, cover a considerably broader pH range.

*Hydrogen-bond-breaking reagents*

*Potassium thiocyanate.* KSCN (4M) was added to soluble eucollagen in 0·1M-citric acid, pH 2·2, to give concentrations up to 0·8M. All the systems remained at pH 2·2. It was clear from the fibre formation that transition to gelatin had not taken place and there was no indication of such a change up to 7 days. The turbidity–KSCN concentration relationship is shown in Fig. 2. A steep increase in turbidity was observed from 0·2M- to 0·4M-KSCN followed by a gradual turbidity decrease between 0·4M- and 0·8M-KSCN.

It is probable that pH 2·2 represents a favourable condition for demonstrating KSCN-induced turbidity. The maximum turbidity effect at this pH value was obtained with 0·4M-KSCN and this concentration was used to demonstrate declining turbidity with increasing pH, obtained by additions of N-NaOH up to pH 5. The results are shown in Fig. 2. In this instance, a steep fall in turbidity occurs in the range pH 4–4·6, quite contrary to the effect when KSCN is absent. This turbidity change with pH is reversible in both cases.

*Sodium salicylate.* The addition of small quantities of 4M-sodium salicylate to soluble eucollagen in 0·1M-citric acid resulted in sharp turbidity changes. The turbidity value reached a maximum at 0·12M-salicylate, at which concentration the system was at pH 3·9. Further additions of salicylate caused the turbidity to diminish so that at 0·4M and pH 4·3 the solution had almost cleared (see Fig. 2). If a further small volume of 4M-sodium salicylate was added and the mixture kept for a few minutes, a felted mass of salicylic acid in characteristic needle-shaped crystals slowly deposited. This last stage demonstrated that, with the higher concentration of sodium salicylate, the soluble eucollagen had the property of behaving as a protective colloid so that the slow crystallization of salicylic acid resulted. The addition of small quantities of 4M-sodium salicylate to 0·1M-citric acid in the absence of soluble eucollagen resulted in the immediate precipitation of salicylic acid.

*Fibre formation with sodium sulphate and chondroitin sulphate.* Fibrous precipitates are formed from acid solutions of soluble eucollagen by NaCl and $Na_2SO_4$ when the solution is below pH 5. The difficulty experienced in washing a precipitate free from $Na_2SO_4$ suggests a degree of ionic complex formation. Fibrous precipitates were obtained with an osseomucoid preparation (Courts, 1959) and chondroitin sulphate below pH 5. The latter precipitate appeared to be a complex of approximately equal proportions by weight of soluble eucollagen and chondroitin sulphate.

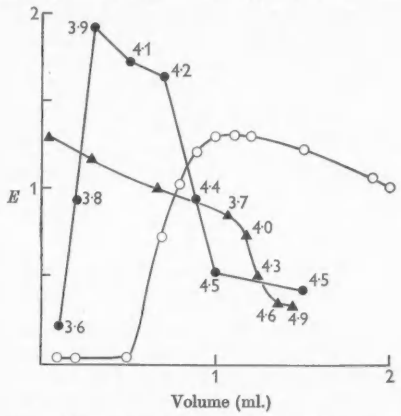


Fig. 2. Turbidity changes of soluble eucollagen in 0·1 M-citric acid with added reagents: ○, 4 M-KSCN: ▲, 0·4 M-KSCN, soluble eucollagen system altered with N-NaOH; ●, 4 M-sodium salicylate. The numbers alongside the curves show the pH at the points indicated; with 4 M-KSCN pH 2·2 was maintained throughout.

*Solubilization of collagen with hydrogen peroxide*

*Treatment at 21°, with hydrogen-bond-breaking reagents.* Only negligible amounts of collagenous protein were dissolved by 1·7 M-$H_2O_2$ at 21°. The collagen–$H_2O_2$ system equilibrated at pH 5·5. The same treatment had very limited action in converting the collagen into eucollagen since only 9 %, washed free from $H_2O_2$, was converted into gelatin at 60° in 3½ hr. Certain of the highly active hydrogen-bond-breaking reagents were more successful in solubilizing collagen, particularly 4 M-LiBr (see Table 3), but, as already indicated by Courts (1958), 8 M-urea is comparatively ineffective.

The presence of 1·7 M-$H_2O_2$ with all the hydrogen-bond-breaking reagents examined had a marked effect on collagen solubilization and, with sodium salicylate, almost complete conversion into gelatin was obtained within 4 weeks (Table 3). The pH value of 1·7 M-$H_2O_2$ was altered by the addition of 8 M-urea or 4 M-sodium salicylate, becoming pH 8 in both instances.

*Treatment at 60°.* When the reaction with 1·7 M-$H_2O_2$ was performed at 60°, collagen was converted into gelatin, the amount converted being proportional to the time of heating. The gelatin, once formed, was then subject to degradation at 60° by the $H_2O_2$. The collagenous residue washed free from $H_2O_2$ yielded further quantities of gelatin, either in water at 60° or in 2 M-sodium salicylate at 18°. The gelatins obtained during the second extraction generally showed superior physical properties to those obtained in the presence of $H_2O_2$. The yield and properties are given in Table 4. The presence of $Na_2SO_4$ completely inhibited the transformation of collagen into gelatin in 1·7 M-$H_2O_2$ at 60°. This was shown in both the 3½ and 4½ hr.

Table 3. *Effect of 1·7 M-hydrogen peroxide on the solution of collagen in lyotropic salts at 21°*

| Reagent | Collagen dissolved by the reagent (%) | | Gelatin viscosity obtained via reagent in $H_2O_2$ (cP) |
|---|---|---|---|
| | In water | In $H_2O_2$ | |
| 4 M-LiBr | 17 | 48 | 3·5 |
| 4 M-Sodium salicylate | 3 | 80 | 4·1 |
| 8 M-$CaCl_2$ | 3 | 53 | 4·2 |
| 8 M-Urea | 1 | 58 | — |
| 1·7 M-$H_2O_2$ | 1 | — | — |
| 8 M-Urea + M-citric acid | 4 | — | — |

Table 4. *Properties of peroxide precursor gelatins and controls*

1st extractions were made in 1·7 M-$H_2O_2$, 60° for the indicated times; 2nd extractions were made in 4 M-sodium salicylate at 20° for 1 day.

| | 2½ hr. | | 3 hr. | | 3½ hr. | | 3 hr., 60° | 3½ hr., 60° |
|---|---|---|---|---|---|---|---|---|
| | 1st | 2nd | 1st | 2nd | 1st | 2nd | Water | 0·1 N-HCl |
| Gelatin yield (%) | 22 | 18 | 36 | 17 | 49 | 20 | 2 | 30 |
| Viscosity (cP) | 3·8 | 6·8 | 3·2 | 7·3 | 2·8 | 6·3 | — | 3·7 |
| Gel strength (g. Bloom) | 179 | 203 | 188 | 206 | 152 | 185 | — | 230 |
| $H_2O_2$ content (%, w/v) | 0·7 | Nil | 0·8 | Nil | 0·8 | Nil | — | — |

Table 5. *N-Terminal amino acid residues of heated collagen*

Measurements were made on washed, reagent-free samples. Values are expressed as mole/100 000 g. of collagen.

| | Treatment | | | | |
|---|---|---|---|---|---|
| | 1·7 M-$H_2O_2$ 2 hr., 60° | $H_2O_2$ then water 2½ hr., 60° | Water 3 hr., 60° | 0·14 M-$H_2SO_4$–$Na_2SO_4$, 80° 40 min.* | Soluble eucollagen, citrate extract |
| Glycine | 0·21 | 0·23 | 0·18 | 0·35 | 0·86 |
| Serine | 0·08 | 0·10 | 0·04 | 0·05 | 0·16 |
| Threonine | 0·08 | 0·13 | 0·02 | 0·08 | 0·12 |
| Alanine | 0·07 | 0·05 | 0·00 | 0·10 | 0·10 |
| Aspartic acid | 0·06 | 0·06 | 0·03 | 0·05 | 0·14 |
| Glutamic acid | 0·11 | 0·10 | 0·01 | 0·08 | 0·13 |
| Others | 0·10 | 0·05 | 0·00 | 0·05 | 0·10 |
| Total | 0·71 | 0·72 | 0·28 | 0·76 | 1·62 |
| $10^{-3}C_n$ | 139 | 138 | 350 | 132 | 62 |

* From Courts (1960).

Table 6. *Effect of peroxide on lyotropic action in gels*

| Reagent | Time for complete gel liquefaction at 18° (min.) In water | In 1·7 M-$H_2O_2$ |
|---|---|---|
| None | >2000 | >2000 |
| 2 M-Urea | >2000 | 12 |
| M-NaBr | >2000 | 30 |
| 2 M-NaBr | 20 | 4 |
| 0·5 M-Sodium salicylate | >2000 | 35 |
| 0·7 M-Sodium salicylate | 80 | 15 |
| 0·9 M-Sodium salicylate | 18 | 8 |

reactions. The yield of gelatin was less than 1% compared with 54 and 61% respectively when $Na_2SO_4$ was absent.

Analyses of the *N*-terminal amino acid residues were made on the insoluble fraction of collagen after heating in 1·7 M-$H_2O_2$ at 60° and the insoluble fraction after further heating in water at 60°. The fractions gave results (see Table 5) equivalent to $C_n$ 139 000 and $C_n$ 138 000 respectively, values which closely resemble the $C_n$ 132 000 for a similar collagen heated in 0·4 N-$H_2SO_4$ in the presence of $Na_2SO_4$ (Courts, 1960).

In a gelatin prepared by the action of 1·7 M-$H_2O_2$ on collagen at 60° (Table 1), certain amino acids appear to have been affected by $H_2O_2$ treatment. Thus the methionine content has been considerably decreased and the proportion of histidine is also lower. On a gram-residue basis both of these decreases are equivalent to 4½ and 3½ g.residues/$10^5$ g. of gelatin respectively. The hydroxy amino acids have higher contents, serine increases by 7 and threonine by 4½ g.residues/$10^5$ g. of gelatin.

Of those amino acids that were subjected to 1·7 M-$H_2O_2$ at 60°, no alteration could be detected by paper chromatography with glycine, serine, proline, hydroxyproline or aspartic acid. Glycylglycine was also unaffected. On the other hand, methionine completely disappeared and was replaced by a spot corresponding to threonine. The histidine spot was reduced in intensity when compared with the control and a second spot corresponding to serine accompanied the histidine on the chromatogram.

*Effect of peroxide on gelation*

Table 6 shows that where a 'weakly active' hydrogen-bond-breaking reagent is used at too low a concentration, the liquefying effect on a gelatin gel is negligible. The addition of $H_2O_2$ greatly increased the efficiency of the lyotropic reagents. This was also seen with 2 M-NaBr which liquefied the standard gel five times more quickly in the presence of 1·7 M-$H_2O_2$. No lyotropic action could be detected with $H_2O_2$ alone up to 4 M.

## DISCUSSION

Two soluble fractions that are fundamentally different in character may be derived from collagen. These are gelatin and acid-soluble collagen. The conversion of ox-bone collagen ($C_n$ 350 000) into eucollagen ($C_n$ 62 000) enhanced the gelatin yield from 2 to 100% (as defined by Courts, 1960) and the dispersion into 0·1 M-citric acid from about 0·2 to about 30%. When eucollagen is solubilized no significant change in $C_n$ occurs whether the resultant form is gelatin or soluble eucollagen and hence no primary bonds are broken in either process. This may be illustrated by:

Eucollagen, $C_n$ 62 000 → solution with disorientation → gelatin, $C_n$ 60 000
Eucollagen, $C_n$ 62 000 → solution with orientation → soluble eucollagen, $C_n$ 62 000

The enhanced solubility of eucollagen into a fibre-forming material with a reduced specific viscosity equivalent to soluble collagens (Courts &

Stainsby, 1959) suggests the reversion of developed collagen into a type of fundamental unit.

The amino acid composition of soluble eucollagen is in general similar to the analyses obtained by Eastoe (1955) and Eastoe & Leach (1958) for bovine collagens and gelatins. Two differences are of possible significance. The hydroxyproline value at 14·9% is appreciably above that of 14·4% obtained for a purified gelatin (Eastoe, 1955) and higher than the values of 12·6–14·3% generally reported. A hydroxyproline value of 14·9% has recently been reported by Eastoe (1960) for a gelatin that had received an unusual pretreatment. The implications that a high hydroxyproline content indicates a high degree of purity in the collagen group of proteins has been discussed by Courts & Kragh (1960). Most of the tyrosine found in native collagen (about 1%) may be regarded as contributed by the non-collagen impurities and this is indicated by the appreciably lower tyrosine values reported for gelatin (0·2–0·6%) (Bowes, Elliott & Moss, 1953; Eastoe & Leach, 1958). However, Gross (1957) finds that dissolved collagens always have at least 0·3% of tyrosine, so that the low tyrosine value of 0·15% for soluble eucollagen would suggest it to be particularly free from impurity. Although the methods used in the preparation of soluble eucollagen are likely to be very effective in removing extraneous materials, the method might have given rise to a collagen fraction with an atypical amino acid composition.

The hydrothermal conversion of soluble eucollagen fibres into gelatin involves two stages. The first is a sharp-melting phenomenon. At this stage, the inability of the 43° melt to gel and the high viscosity of its citric acid solution (Gallop, 1955; Doty & Nishihara, 1958) indicates that the molecular organization associated with soluble collagens has not been disrupted. The interchain forces disrupted are likely to be of the van der Waals type and this change is mainly reversible.

The second stage at 44° is molecular disorientation and transition to gelatin. This change is for the most part irreversible. Although these observations were made on reconstituted eucollagen fibres, in which water represents about 95% of the total mass, it is possible that similar, but less well-defined, stages occur with native collagen. Flory & Garrett (1958) and Witnauer & Fee (1959) regard the shrinkage of collagen to be purely a melting phenomenon. Witnauer & Fee (1959) have predicted that, if melting is the uncoiling of helices, then the temperature for the shrinkage of intact collagen in water should be very nearly that for soluble collagen (i.e. isolated helices) at the same pH. This view is exactly confirmed with eucollagen in which the shrinkage temperature of 44° in water (Courts, 1960) is the same as that of the reconstituted soluble eucollagen at the same pH. However, the initial melting of the reconstituted fibres at 43° cannot be regarded, by itself, as the uncoiling of the collagen helix. Any subsequent molecular disorientation is likely to be strongly dependent upon environment and, as such, this stage would be the rate process in native collagen, which Weir (1949) suggested.

The development of turbidity in citrate extracts of eucollagen between pH 4 and 5 is best explained as isoelectric behaviour. The production of maximum turbidity at pH 4·33 (± 0·03) would therefore correspond with the isoelectric point (pE). The shift, $pI - pE = -0{\cdot}5$, which has been induced by citrate ions (or molecules) is small compared with the value of −4 determined by Jackson & Neuberger (1957) for acid-processed gelatins. The small shift is, however, predictable from the known strong buffering capacity of alkali-processed gelatin (p$I$ about 4·8) in the isoelectric region (Kenchington & Ward, 1954) where addition or removal of hydrogen ions incurs a relatively small pH change.

The precipitation of fibres of soluble eucollagen by dilute forms of hydrogen-bond-breaking reagents is of some interest, since similar quantities incorporated into a gelatin gel, a largely hydrogen-bonded system, would interfere with gelation. Thus, even in a concentrated (5·65%, w/v) gel, 0·1 M-sodium salicylate reduced the gel strength by 17% and 0·1 M-potassium thiocyanate reduced the strength of the same gel by 10% (A. Courts, unpublished work). The turbidity induced by potassium thiocyanate in soluble eucollagen is somewhat different from isoelectric behaviour and the charge distribution along the protein molecule is unaltered. About half the molar concentration of salicylate is required to produce the same maximum turbidity effect as potassium thiocyanate. A similar relationship occurs in the results of Brown *et al.* (1958) in $T_s$ depression studies. The reconstitution of fibres from solubilized collagens by salicylates or thiocyanates has not previously been reported. The ability of soluble eucollagen to reconstitute as fibres by dialysis, by salting out and by forming complexes with chondroitin sulphate indicates a structural similarity with soluble collagens, in support of the reduced specific viscosity data of Courts & Stainsby (1959). The electron micrographs of reconstituted soluble eucollagen from human bone (Little *et al.* 1961) did not reveal any banded structure.

Reactions between proteins of the collagen group and 'oxidizing agents' has been studied in most detail with sodium periodate (Desnuelle & Antonin, 1946; Grassmann & Kuhn, 1955; Gustavson, 1958; Hörmann & Fries, 1958; Hörmann, 1960). Investigations with hydrogen

peroxide appear to be more limited. Orekhovich & Shpikiter (1957) state that 6M-hydrogen peroxide irreversibly modifies procollagen into simpler components which may be related to gelatin. There appears to be little evidence to indicate that changes that these 'oxidizing agents' bring about in collagen are due to oxidation of the molecule or that periodate and peroxide behave in the same way.

Bovine collagen is not solubilized in 1·7M-hydrogen peroxide at 20°, but undergoes extensive solubilization when a hydrogen-bond-breaking mechanism is used in conjunction with the peroxide. This mechanism can involve either heat or certain lyotropic reagents. In both instances the collagen is converted into gelatin. It appears likely that the course of this conversion is, in the initial stages, similar to the collagen into eucollagen reaction (Courts, 1960) as instanced by the substantial gel-forming and viscometric properties. It is interesting that, whereas in the presence of 0·7M-hydrogen peroxide at 60°, gelatin has undergone some secondary degradation, the collagenous residue has not and this residue undergoes a further conversion into high-grade gelatin in the absence of hydrogen peroxide, either in water at 60° or in 2M-sodium salicylate at 18°. This reaction presents further evidence that eucollagen can retain molecular integrity through hydrogen bonding against a reagent that will degrade the molecularly disorientated form, gelatin. This was implied by Courts (1960) in connexion with acids and alkalis. The collagen–1·7M-hydrogen peroxide system equilibrates at pH 5·5. When 8M-urea was introduced to lead to extensive solubilization of the collagen the system was at pH 8. When 4M-sodium salicylate was used to solubilize the collagen, the system was again pH 8. It is considered unlikely that hydrogen ions play a major part in these reactions since the pH of the systems is generally near neutrality. Hörmann (1960) has reported the dissolution of collagen by hydroxylamine in conjunction with lithium bromide or potassium thiocyanate, but not alone.

Kalyankar, Vaidyanathan & Giri (1955) have studied the action of 0·1M-hydrogen peroxide on certain amino acids in the presence of iron salts at 25°. They explain their results in terms of the dissociation of peroxide as free radicals (Haber & Weiss, 1934). The disorientation of collagen by a hydrogen-bond-breaking mechanism induced by free hydroxyl radicals would be a useful model to collate the apparently parallel reactions of collagen with hydrogen peroxide and hydroxylamine in the presence of lyotrophic salts and of sodium periodate and hydrogen peroxide at elevated temperatures.

Little *et al.* (1961) have investigated by electron microscope the irradiated collagen described by Bowes *et al.* (1960). With hydrated material, the fibre bundles coalesce and become structureless. The production of free hydroxyl radicals in aqueous systems by ionizing radiation points to an analogy with the mechanism proposed for the collagen–peroxide system.

## SUMMARY

1. Controlled alterations to the structure of bone collagen by alkali has led to increased solubility in acid buffer solutions, resembling a reversion of the developing processes.

2. In this solubilized form, the collagen-type structure has been maintained, as shown, for example, by the ability to re-form fibres. After its derivation from eucollagen, this form is termed 'soluble eucollagen'.

3. Soluble eucollagen was isoionic at pH 4·8 and gave an ultimate isoelectric point at pH 4·3 in the presence of acetate, citrate, lactate and mandelate solutions.

4. The amino acid analysis indicated a typical collagen of high purity.

5. Hydrothermal melting and disorientation of reconstituted soluble eucollagen occurred in two distinct stages.

6. Hydrogen peroxide dissolved intact collagen at 60° to give, initially, high-molecular-weight fragments. Only limited oxidation occurred.

7. Hydrogen peroxide catalysed the conversion of collagen into gelatin at 21° by hydrogen-bond-breaking reagents, e.g. sodium salicylate.

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# The Action of Anions on Catalase Peroxide Compounds

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Catalase, like other haemoproteins, shows two kinds of chemical behaviour in respect of its haem iron, namely complex-formation and oxidation–reduction. The role of these two types of reaction in the catalytic activity (both catalatic and peroxidatic) of this enzyme has been the subject of some controversy. According to Chance (cf. Chance & Fergusson, 1954), the intermediates involved in the catalytic reactions are essentially complexes (similar to the fluoride or cyanide derivatives); according to George (1952, 1953*a*), they should rather be considered as higher oxidation states.

The chemical nature of these intermediates (catalase peroxide compounds I and II) can be most usefully investigated by examining the reactions they undergo. The best-known group of such reactions comprises those with hydrogen donors, the behaviour of which has been analysed in terms of six reaction types (Keilin & Nicholls, 1958; Nicholls, 1959). These six types of donor

each promote one or more of several reduction steps leading from the primary peroxide compound (compound I) back to the free catalase (ferric). The absorption spectra of the two chief catalytic intermediates, compound I (containing two oxidizing equivalents in excess of the ferric state) and compound II (with one such oxidizing equivalent) are markedly different. But the two one-electron reductions, that of compound I to II and that of compound II to the free ferric catalase, show certain similarities. For example, the effects of pH are identical, and the reactions with phenols are in each case second-order (Nicholls, 1959). The two processes are also very similar to the corresponding reactions in peroxidase (George, 1952; Keilin & Nicholls, 1958).

Of the reactions given in Fig. 1, the present paper is largely concerned with the two one-electron reduction steps. Unlike the other reactions (promoted by donors in groups $D_3$, $D_5$ and $D_6$), these reductions are also produced by a hydrogen donor endogenous to the catalase protein itself, as with peroxidase.

The question of the relation between the redox reactions of the enzyme and its ability to form reversible complexes in the ferric form with anionic ligands, will also be considered. It has long been recognized that the inhibition of catalase by such ligands can be interpreted in terms of complex-formation with the free ferric enzyme (Keilin & Hartree, 1935). Such spectroscopically distinct complexes are closely similar to those formed by methaemoglobin and peroxidase. For catalase, the proposed mechanism is (Chance, 1952*b*):

$$\text{Catalase}\cdot Fe^{3+}\cdot H_2O + HA \rightleftharpoons \text{Catalase}\cdot Fe^{3+}\cdot HA + H_2O \quad (1)$$

(HA: undissociated acid)

Nevertheless, the action of some anions on the catalatic activity of the enzyme cannot be accounted for by this simple scheme. Agner & Theorell (1946) found that the inhibition of catalase activity by acetate does not correspond with the spectrophotometric dissociation curve for enzyme and inhibitor. Bonnichsen, Chance & Theorell (1947) demonstrated a progressive inhibition of catalase by acetate (but not by formate or phosphate) in the presence of peroxide. Ogura, Tonomura, Hino & Tamiya (1950) showed that such inhibition occurs in the presence of phenols and various complex-forming anions, except cyanide. Beers & Sizer (1954, 1956) confirmed these observations with respect to acetate, chloride, azide and sulphide, and suggested that rapid formation of the secondary peroxide compounds is involved.

Each of these scattered observations suggests that other reactions between anions and enzyme, besides reaction (1), occur during the catalatic reactions. They further suggest that the reactions concerned promote the formation of essentially irreversibly inhibited derivatives of catalase, in contrast with the typically reversible inhibition of reaction (1). These ideas are supported to some extent by an analogy with peroxidase. The existence of an endogenous hydrogen donor in peroxidase preparations is well known (Chance, 1949*a*; George, 1953*b*). It is responsible for the 'spontaneous' transition from compound I to compound II, and for the decomposition of compound II. Chance (1949*a*, 1952*c*) and George (1953*b*) have shown that the decomposition of compound II by this donor (although not the formation of compound II from compound I) is promoted by complex-forming ligands such as acetate, fluoride and cyanide. There is some doubt about the order of the reaction with respect to fluoride concentration (Chance, 1952*c*; George, 1953*b*). Nevertheless, the reaction shows that complex-forming anions can react with derivatives other than the free enzyme.

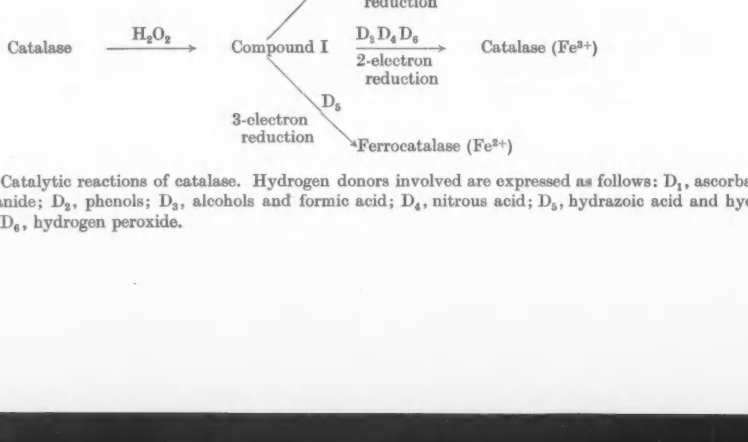


Fig. 1. Catalytic reactions of catalase. Hydrogen donors involved are expressed as follows: $D_1$, ascorbate and ferrocyanide; $D_2$, phenols; $D_3$, alcohols and formic acid; $D_4$, nitrous acid; $D_5$, hydrazoic acid and hydroxylamine; $D_6$, hydrogen peroxide.

The demonstration of the analogy between compounds II of catalase and peroxidase (George, 1952; Keilin & Nicholls, 1958) shows that the transitions producing and decomposing the secondary compound of catalase (Chance, 1950) must also be due to the 'endogenous donor'. As the secondary compound is catalatically inactive, its formation and steady state will influence markedly the observed kinetics of the catalatic reaction. It is thus possible that the anomalies observed in the inhibition of catalase are associated with an effect of anions on the redox reactions of the enzyme (i.e. the formation and decomposition of compounds I and II) and, in particular, those reactions in which the hydrogen donor is endogenous.

This paper shows that the reductions produced by this endogenous process are different in kind from the reductions by exogenous donors, and that the kinetics of the former reductions may be used to demonstrate a reaction between complex-forming ligands and the catalase peroxide compounds themselves.

## METHODS AND MATERIALS

*Catalase.* Catalase was prepared from horse liver (method of Keilin & Hartree, 1945) or horse blood by a modification of the methods of Bonnichsen (1955) and Herbert & Pinsent (1948). In all experiments, the formation and destruction of the secondary peroxide compound was followed qualitatively by direct visual observations with a bench microspectroscope. Quantitative measurements were made with a spectrophotometer containing a glass-prism monochromator, giving high dispersion in the visible region of the spectrum. The kinetics of compound II were followed by fixing the wavelength at 565 mμ, which is close to the absorption maximum of compound II and which is also effectively isosbestic for compound I and free catalase.

*Manometry.* Barcroft–Warburg microdifferential manometers were used, at 30°, with air as the gas phase.

*Other materials.* These included: solutions of notatin (glucose oxidase) prepared from a purified sample of the enzyme (supplied by Boots Pure Drug Co. Ltd.); ethyl hydroperoxide, made according to the method of Baeyer & Villiger (1901); sperm-whale metmyoglobin solutions were prepared from a crystalline sample of myoglobin kindly supplied by Dr J. C. Kendrew. All other reagents used were of AnalaR or equivalent quality.

## RESULTS

### *Formation of catalase peroxide compound II and the endogenous donor*

The addition of notatin (glucose oxidase) and glucose, followed by aeration, which generates $H_2O_2$, or of ethyl hydroperoxide to a solution of catalase, results in the production of compound II, with absorption bands at 568 and 535 mμ. Fig. 2 shows the changes in extinction that occur in acetate and phosphate buffers. Acetate but not phosphate is a ligand forming a complex with catalase, and its effect on the system is considered in detail below. The true decomposition rate of compound II can be measured only if the steady-state compound I is removed, e.g. by the addition of ethanol, which decomposes compound I but not compound II (Keilin & Nicholls, 1958). Fig. 3 shows the effect of pH on the formation and decomposition of compound II by this method, and also its formation by ethyl hydroperoxide (cf. Chance, 1952*a*). The rate of formation is approximately four times as great in the latter case, because only one-quarter of the haems are bound as compound I by the hydrogen peroxide generated in the notatin system (Chance, 1950).

Table 1 shows the relationship between catalase concentration and the velocity constant for these one-electron transitions.

Unlike the results obtained by Fraser (1956) for the two-electron reduction of compound I to free catalase, the velocity of which depended on the square of the enzyme concentration, the transition from compound I to compound II is first-order with respect to catalase concentration. This indicates that the reducing substance involved is associated with the same protein molecule as the haem. Further, there is no consistent method of titrating the endogenous donor with an oxidizing agent to decrease the velocity constants for the

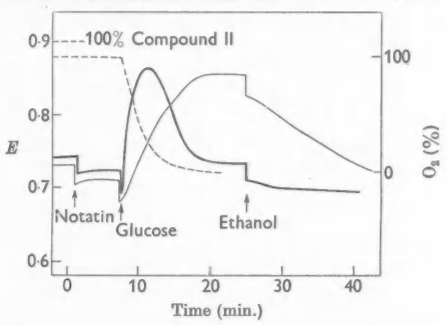


Fig. 2. Formation of catalase compound II by hydrogen peroxide generated in the notatin–glucose system, and its subsequent decomposition. Notatin (10 μg.) and 1 mg. of glucose were added to 2·7 ml. of aerated catalase solution (0·06 mM-liver-catalase haem). The formation of compound II is followed by spectrophotometric measurements at 565 mμ: —, in 0·05 M-acetate buffer, pH 4·6; —, in 0·1 M-acid phosphate, pH 5·0; - - - -, concentration of oxygen calculated from manometric experiments in phosphate buffer, from the kinetic data of Keilin & Hartree (1948) and Laser (1952). 100% Oxygen indicates saturation with air (about 0·2 mM-$O_2$ at 20°). '100% Compound II' indicates $E$ at 565 mμ in the presence of ferrocyanide, which reduces compound I to compound II without affecting the stability of the latter.

transitions. Incubation with ferricyanide had no effect whatever on the formation of compound II. Incubation with ethyl hydroperoxide affected the steady state of compound II produced in the notatin system, but this effect was largely due to an increase in the rate of decomposition of compound II, and was accompanied by the destruction of part of the enzyme protein.

A similar decrease in the stability of compound II was found in certain samples of catalase that had undergone partial denaturation, such as 'para-catalase' (cf. Keilin & Hartree, 1951) and reducible catalase (cf. Dounce & Schwalenberg, 1950). The effect of ethyl hydroperoxide may therefore be due to alteration in the availability of the endogenous donor, rather than to its saturation with oxidizing equivalents. In respect of this lack of titratability, the donor resembles the endogenous donor in peroxidase (Chance, 1949*a*) and differs markedly from all added donors.

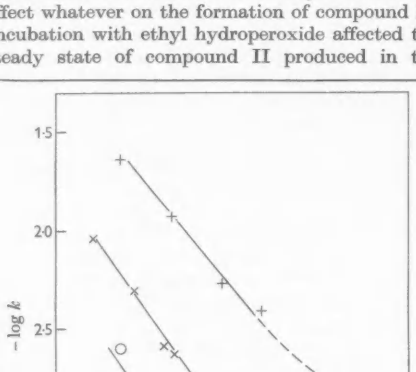


Fig. 3. Effect of pH on the rates of formation and decomposition of compound II. +, Rate of production of compound II from ethyl hydroperoxide compound I; ×, rate of production of compound II from the compound I steady state in the presence of hydrogen peroxide generated by the notatin–glucose system; ○, rate of decomposition of compound II induced by addition of ethanol to remove compound I. 20 $\mu$M-Catalase; 2 $\mu$moles of peroxide was added to 2·7 ml. of solution in ethyl hydroperoxide experiments; 10 $\mu$g. of notatin and 1 mg. of glucose were added to 2·7 ml. of aerated solution in the notatin experiments; 10 $\mu$moles of ethanol was used to promote decomposition; phosphate buffers; 20°. $k$ is in each case a first-order constant (with the dimensions sec.$^{-1}$).

Table 1. *Velocity constants for compound II formation at different concentrations of catalase*

The constants were obtained under the following conditions: 0·1 M-phosphate buffer, pH as indicated, at 20°. Notatin (10 $\mu$g.) with 1 mg. of glucose was added in some experiments, 2 $\mu$moles of ethyl hydroperoxide in others. The final reaction volume in all cases was 2·7 ml., and measurements were made at 565 m$\mu$. The constant $k$, measured in reciprocal seconds, is the apparent first-order velocity constant for the formation of compound II from free catalase.

| $10^5$ × Concn. of catalase (M) | Source of peroxide: Ethyl hydroperoxide $10^3 k$ (sec.$^{-1}$) | Ethyl hydroperoxide pH | Notatin system $10^3 k$ (sec.$^{-1}$) | Notatin system pH |
|---|---|---|---|---|
| 1·4 | 3·5 | 5·6 | 1·1 | 5·3 |
| 2·2 | 2·5 | 5·6 | 1·4 | 5·3 |
| 6·0 | 2·7 | 5·6 | 1·2 | 5·3 |

*Action of ligands*

The anions combining with free (ferric) catalase may be divided into three groups: (1) ligands forming ionic complexes (e.g. fluoride, acetate, formate, hypophosphite); (2) ligands forming ionic complexes, also inhibiting by donor reaction (e.g. azide, hydroxylamine); (3) ligands forming covalent complexes (e.g. cyanide, sulphide).

*Normal ionic ligands.* Fluoride and acetate were found to accelerate both the formation and decomposition of catalase compound II (see Fig. 1). In neither case is the effect directly proportional to anion concentration (cf. peroxidase; George, 1953*b*). The efficacy of fluoride proves that the reaction is a catalytic one, as fluoride is thermodynamically incapable of acting as a hydrogen donor itself (unlike acetate and formate).

In Fig. 4 the second-order velocity constants for the formation and decomposition of compound II have been plotted against the logarithm of the fluoride concentration. The results can be fitted to sigmoid curves which are consistent with Michaelis–Menten kinetics. The curves in Fig. 4 are compared with the dissociation curve for fluoride catalase obtained spectrophotometrically at the same pH. Table 2 summarizes the reaction parameters deducible from Fig. 4.

Change in pH has a marked effect on the action of fluoride towards compounds I and II. This is compounded of effects on the maximal velocity constant and on the apparent Michaelis constant. Table 3 illustrates this for pH values 5·0 and 6·3.

As in complex-formation with the free enzyme, the dissociation constants seem to be inversely proportional to $H^+$ ion concentration. As in the spontaneous transition (Fig. 2), the maximal velocity constants increase with $H^+$ ion concentration but are not directly proportional to it. We have here a relation between the action of catalase haem as complex-forming agent for anions and as oxidizing agent for hydrogen donors.

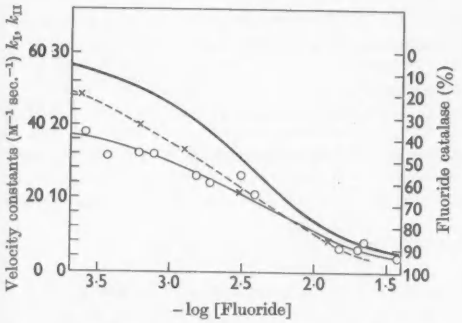


Fig. 4. Variation of velocity constants for the action of fluoride on catalase peroxide compounds compared with the dissociation curve of fluoride catalase. $k_I$ is the second-order velocity constant for the transition between compound I and compound II catalysed by fluoride ($M^{-1}$ sec.$^{-1}$); $k_{II}$ is the corresponding constant for the fluoride-catalysed decomposition of compound II ($M^{-1}$ sec.$^{-1}$). All measurements refer to phosphate, pH 5·0, at 20°. $k_I$ values (×) are given by - - - -, $k_{II}$ values (○) by — and the fluoride-catalase dissociation curve by —. The dissociation curve was obtained by adding appropriate amounts of fluoride to catalase and measuring the appearance of fluoride catalase at 612 mμ. The dissociation constant corresponds closely to that given by Chance (1952*b*).

Table 2. *Reactions of fluoride with catalase*

Compound II was formed by means of the notatin system or ethyl hydroperoxide in 0·1 M-phosphate buffer, pH 5·0, at 20°. The dissociation constants are obtained from Fig. 4.

| Reaction | Velocity constant (sec.$^{-1}$): Fluoride absent | Velocity constant (sec.$^{-1}$): Excess of fluoride | Apparent dissociation constant (mM) |
|---|---|---|---|
| Fluorcatalase formation | — | — | 3·0 |
| Formation of compound II | 0·012 | 0·15 | 3·3 |
| Decomposition of compound II | 0·0009 | 0·08 | 4·4 |

Acetate, the action of which is shown in Fig. 1, behaves in a manner similar to fluoride. Formate acts likewise in the decomposition of compound II, but reacts with compound I as two-electron hydrogen donor (Keilin & Nicholls, 1958). Kinetic constants for these anions are given in Fig. 5 and Table 5.

That this effect is associated with the peroxide compound itself, and not a reactive equilibrium form in low concentration, is shown by the use of sodium hypophosphite. Unlike the other ionic ligands, its reaction with catalase is very slow, although the resultant complex has a typical ionic spectrum.

$$\text{Catalase}\cdot Fe^{3+} + H_2O + H_3PO_2 \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} \text{catalase}\cdot Fe^{3+}\,H_3PO_2 + H_2O$$

$k_9 = 0{\cdot}35\,M^{-1}$ sec.$^{-1}$ (pH 4·8)

$k_9'$ (undissociated acid, p$K$ 1·7) $= 440\,M^{-1}$ sec.$^{-1}$

$k_{10} \leqslant 10^{-6}$ sec.$^{-1}$ (dialysis for 3 days removed only a small percentage of the hypophosphite complex).

Hypophosphite induces the decomposition of compound II, with the concomitant formation of hypophosphite–catalase. At low hypophosphite concentrations, the formation of bound enzyme from compound II in this way is as fast as from free enzyme. At high hypophosphite concentrations, the velocity reaches a limiting value of the same order of magnitude as with other anions. $K_m$ corresponds to the ratio of the rates of formation and decomposition of a hypothetical anion–compound II complex spectroscopically indistinguishable from compound II itself, as shown in the following calculation.

$$\text{Catalase compound II} + HA \underset{k_3}{\overset{k_2}{\rightleftharpoons}} \text{catalase compound II}\cdot HA \quad (3)$$

$$\text{Catalase compound II}\cdot HA \xrightarrow[\text{endogenous donor}]{k_4} \text{catalase}\cdot Fe^{3+}\,HA. \quad (4)$$

If $v$ represents the experimentally observable rate of disappearance of compound II (M sec.$^{-1}$),

$$v = k_4[\text{compound II}\cdot HA] = \frac{k_4[HA]\,[II]_{total}}{\frac{k_4+k_3}{k_2}+[HA]} \quad (5)$$

where for fluoride, acetate etc. $k_3 \gg k_4$ and $K_m = k_3/k_2$, but for hypophosphite $k_3 \ll k_4$ and $K_m = k_4/k_2$. As described above, for hypophosphite, $k_2 = k_9 = 0{\cdot}35\,M^{-1}$ sec.$^{-1}$ at pH 4·8;

$$k_4 = 4 \times 10^{-2}\ \text{sec.}^{-1}$$

at this pH; $K_m$ can therefore be calculated to be 0·12 M, which corresponds with the value obtained experimentally.

Although anions thus have a marked effect on the reactivity of endogenous donor, no action in reductions by exogenous donors could be detected. The decomposition of compound II by a mixture of fluoride and *p*-cresol was no faster than was predictable on the basis of additive effect. Further, as shown in Table 4, when peroxide is generated by glucose oxidase, the percentage that reacts with hydrogen donors ('percentage utilization' in the

Table 3. *Effect of* pH *on catalysis by fluoride*

Compound II was formed by hydrogen peroxide generated in the notatin system, with 0·04 M-phosphate buffer at 20°.

$pK_d$ Negative logarithm of the dissociation constant for fluoride catalase.

$pK_m^{I}$ Negative logarithm of the Michaelis constant for the decomposition of compound I by fluoride (i.e. formation of compound II, cf. Fig. 4 and Table 2).

$pK_m^{II}$ Negative logarithm of the Michaelis constant for the decomposition of compound II by fluoride (cf. Fig. 4 and Table 2).

$pk_{I\ max.}$ Negative logarithm of the maximal velocity constant for the transition from compound I to compound II (cf. Fig. 4 and Table 2).

$pk_{II\ max.}$ Negative logarithm of the maximal velocity constant for the decomposition of compound II (cf. Fig. 4 and Table 2).

$K_d$, $K_m^{I}$, $K_m^{II}$ are expressed in terms of fluoride concentration (molar); $k_{I\ max.}$, $k_{II\ max.}$ in reciprocal seconds (sec.$^{-1}$).

| pH | $pK_d$ | $pK_m^{I}$ | $pK_m^{II}$ | $pk_{I\ max.}$ | $pk_{II\ max.}$ |
|---|---|---|---|---|---|
| 5·0 | 2·5 | 2·5 | 2·4 | 0·8 | 1·1 |
| 6·3 | 1·2 | 1·3 | 1·0 | 1·4 | 1·6 |

Table 4. *Effect of anions on the peroxidatic utilization of hydrogen peroxide in the coupled oxidation system*

Notatin (20 $\mu$g.) and 10 $\mu$moles of glucose were placed in 1·0 ml. (final volume) of 0·02 M-phosphate buffer, pH 6·0, with the use of microdifferential manometers without alkali papers, at 30°. Catalase, donor and anion concentrations were as indicated. The percentage utilization of $H_2O_2$ was calculated from excess of oxygen uptake compared with the system in the absence of hydrogen donors.

| Concn. of catalase ($\mu$M) | Concn. of donor (mM) | | Concn. of anion (mM) | | Utilization of $H_2O_2$* (%) Anion absent | Anion present |
|---|---|---|---|---|---|---|
| 18 | Ferrocyanide | 10 | Acetate | 20 | 16 | 22 |
| 2 | Ethanol | 20 | Fluoride | 1·0 | 65 | 66 |
| 4 | Nitrite | 10 | Fluoride | 1·0 | 77 | 76 |
| 10 | *p*-Cresol | 10 | Fluoride | 1·0 | 40 | 44 |
| 12 | *p*-Cresol | 10 | Fluoride | 10 | 51 | 49 |

* Percentage of $H_2O_2$ used peroxidatically rather than undergoing catalatic decomposition.

terminology of Keilin & Hartree, 1955) is unaffected by the addition of small quantities of anions.

*Ionic ligands also inhibiting by a donor reaction.* Both azide and hydroxylamine react with catalase in the presence of peroxide to give nitric oxide ferrocatalase (Keilin & Hartree, 1954). This is due to a reduction of compound I by the donor (Chance, 1947), thus (cf. Lemberg & Foulkes, 1948):

$$\left.\begin{array}{l} \text{Catalase}\cdot Fe^{3+} + H_2O_2 \rightarrow \text{compound I} + H_2O \quad (a) \\ \text{Compound I} + HN_3 \rightarrow \text{catalase}\cdot Fe^{2+}NO + N_2 + H^+ \quad (b) \\ \text{or} \\ \text{Compound I} + NH_2OH \rightarrow \text{catalase}\cdot Fe^{2+}NO + H^+ + H_2O \quad (c) \end{array}\right\} \quad (6)$$

Hydroxylamine reduces compound II to free ferric catalase. At pH 5, the reaction is first-order with respect to hydroxylamine between 0·03 and 3 mM, the velocity constant being 280 M$^{-1}$ sec.$^{-1}$. Azide also reduces compound II to ferric catalase, but the kinetics are more complicated. As Fig. 5 shows, at low azide concentrations its effect resembles that of other anions, but at high concentrations the decomposition velocity does not approach a limiting value. This suggests that azide reacts with compound II in two ways: (i) catalytically: $K_m = 1{\cdot}1 \times 10^{-6}$ M, $k_{II\ max.} = 0{\cdot}1$ sec.$^{-1}$ (pH 5, 20°); (ii) stoicheiometrically: $k = 1000$ M$^{-1}$ sec.$^{-1}$ (with respect to total azide present; pH 5, 20°). Hydroxylamine, with a much lower affinity, reacts only in the second way.

*Covalent ligands.* Cyanide was found by George (1953*b*) to react with peroxidase compound II in much the same way as fluoride, although the catalytic effect compared with the dissociation constant of cyanperoxidase was less than that with fluoride. Cyanide may, however, be added to a mixture of catalase and compound II in a concentration sufficient to convert all the catalase into cyancatalase without noticeably affecting the stability of compound II (Chance, 1950).

This effect was also found in the course of the present investigation. If, on the other hand, cyanide is added in very high concentration, it also induces the decomposition of compound II, as shown in the relevant curve of Fig. 5. The maximal

velocity constant is of the order of 0·1 sec.$^{-1}$, and $K_m$ at pH 5 is $7 \times 10^{-2}$ M, which is about $10^4$ times the dissociation constant ($K_d$) for cyancatalase. The second-order velocity constant decreases with increasing pH, being 1·5 M$^{-1}$ sec.$^{-1}$ at pH 5·2 and 0·37 M$^{-1}$ sec.$^{-1}$ at pH 6·9.

Another ligand forming a covalent complex with the free enzyme is sulphide. The peculiar reaction of compound II with this reagent is described in the next paper (Nicholls, 1961).

*Comparison with metmyoglobin*

The present results with catalase were compared with those previously obtained by George and by Chance with peroxidase, and experiments with metmyoglobin to detect analogous reactions of anions with metmyoglobin peroxide were carried out. Fluoride has no significant effect on metmyoglobin peroxide either at pH 8·0 or at pH 5·6 (when the compound becomes spontaneously unstable). Assuming that the maximal decomposition rate of metmyoglobin peroxide in the presence of fluoride was $8 \times 10^{-3}$ sec.$^{-1}$, $K_m$ for fluoride at pH 8 was estimated to be greater than 0·6 M, whereas $K_d = 2{\cdot}7 \times 10^{-2}$ M. Cyanide at pH 8·0 produced a decomposition of the metmyoglobin peroxide compound. As with catalase peroxide compounds, the rate was not directly proportional to cyanide concentration; data indicated a maximal velocity of $8 \times 10^{-3}$ sec.$^{-1}$ and $K_m$ $4{\cdot}4 \times 10^{-3}$ M (cf. $K_d = 8 \times 10^{-7}$ M). The maximal velocity is only one-tenth of that found for the decomposition of catalase compound II.

Both $K_m$ values are markedly greater than the $K_d$ values; the greatest difference between catalase and peroxidase, on the one hand, and metmyoglobin, on the other, lies in the absence of marked effect of fluoride on the reactions of the latter.

## DISCUSSION

The observations recorded here depend upon two properties of the catalase peroxide compounds, their reaction with complex-forming anions, and their instability towards the 'endogenous donor'. These two properties are reflected in the data collected in Fig. 5. In fact, as each reaction represents the formation of a complex with ligand by peroxide compound, each curve is sigmoid, with a slope corresponding to the dissociation of 1 mole of anion/mole of compound II; and, except with cyanide, all $K_m$ values approximate to the $K_d$ values for the corresponding anion–enzyme complexes. As each reaction also represents the activity of the endogenous donor, the velocity constants become zero-order at high anion concentrations, except with azide; and the maximal values vary from about 0·04 to 0·10 sec.$^{-1}$ with different anions.

Table 5 summarizes the constants from Fig. 5, together with the relevant data on the reactions with free enzyme.

Each curve therefore obeys an equation of the form of equation (5); the simplest theoretical system producing such an equation is given by the two postulated reactions (3) and (4). Theories which assume that the anions do not react with compound II itself are ruled out by the evidence that the reaction rate of hypophosphite with compound II is the same as with free catalase.

Comparison of these data for catalase with those obtained with peroxidase (George, 1953*b*) shows important differences, in particular a much greater affinity of the catalase peroxide compounds for the complex-forming ligands. These differences are summarized in Table 6.

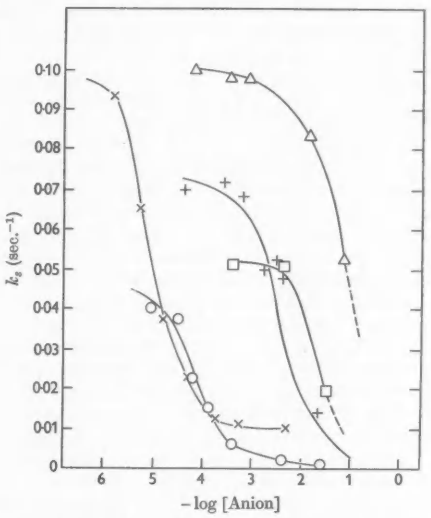


Fig. 5. A comparison of the effects of several anions on catalase compound II: variation of the function $k_s$ with anion concentration, plotted semilogarithmically. The variable $k_s$ is defined at $k_{II} \times K_m$, where $k_{II}$ is the apparent second-order velocity constant for compound II decomposition and $K_m$ is the concentration of anion giving half the maximal rate of compound II decomposition. Theoretically, $k_s$ is related to $k_{max.}$ (Table 5) by the equation $k_s = k_{max.}/(1 + [HA]/K_m)$. Therefore, as the anion concentration approaches zero, $k_s$ approaches $k_{max.}$ (the maximal first-order velocity constant for the decomposition of compound II in the presence of the anion). All curves refer to phosphate, pH 5·0, at 20°: ×, azide; ○, formate; +, fluoride; □, acetate; △, cyanide.

Table 5. *Equilibrium and rate constants for the reactions of anions with free catalase and catalase peroxide compound II*

Values obtained for acetate and formate were with a different set of catalase preparations from those used in the other cases.

| | Free enzyme reaction | | Compound II reaction | | |
|---|---|---|---|---|---|
| Anion | Complex | $K_d$ (M) | $K_m$ (M) | $K_m/K_d$ | $k_{max.}$ (sec.$^{-1}$)* |
| Fluoride | Ionic | $3 \times 10^{-3}$ | $4{\cdot}4 \times 10^{-3}$ | 1·5 | 0·08 |
| Azide | Ionic | $1{\cdot}6 \times 10^{-5}$ | $1{\cdot}1 \times 10^{-5}$ | 0·7 | 0·09† |
| Acetate | Ionic | $1{\cdot}6 \times 10^{-2}$ | $1{\cdot}6 \times 10^{-2}$ | 1·0 | 0·05 |
| Formate | Ionic | $1{\cdot}3 \times 10^{-4}$ | $1{\cdot}3 \times 10^{-4}$ | 1·0 | 0·05 |
| Cyanide | Covalent | $4 \times 10^{-6}$ | $7 \times 10^{-2}$ | $1{\cdot}7 \times 10^{4}$ | 0·10 |

* Values for pH 5·0, 20°. † Excluding donor reaction.

Table 6. *Reactions of two anions, fluoride and cyanide, with catalase, peroxidase and metmyoglobin*

'Active' means active in catalysis of one-electron transition due to endogenous donor.

| | Reaction with fluoride | | | Reaction with cyanide | | |
|---|---|---|---|---|---|---|
| Haemoprotein | Free enzyme | Compound I | Compound II | Free enzyme | Compound I | Compound II |
| Catalase (pH 5) | Ionic (HF only) | Active $K_m = K_d$ | Active $K_m = K_d$ | Covalent (HCN only) | ? | Active $K_m \gg K_d$ |
| Peroxidase (pH 5·4) | Ionic (HF only) | Inactive | Active $K_m > K_d$ | Covalent (HCN only) | Inactive | Active $K_m > K_d$ |
| Metmyoglobin (pH 8·0) | Ionic ($F^-$ and HF react) | — | Inactive | Covalent ($CN^-$ and HCN react) | — | Weakly active $K_m > K_d$ |

The effects of anions on the catalase peroxide compounds, described above, are of the kind required to account for the anomalies found in the inhibition of catalase by such ligands, mentioned in the introductory paragraphs. Further evidence is thus supplied for the view that all the characteristics of the overall catalatic reaction can be interpreted in terms of reactions of the peroxide compounds. In the present case, such an interpretation is shown to be possible for the progressive and effectively irreversible inhibition occurring in the presence of anions during the decomposition of hydrogen peroxide.

The present results also suggest an answer to the question why the reaction of ethyl hydroperoxide with catalase to form compound II is not independent of the alkyl peroxide concentration (see Chance, 1949*b*) as it is with peroxidase (Chance, 1949*a*). Fraser (1956) proposes a reaction of the alkyl peroxide as a hydrogen donor towards compound I:

$$\text{Compound I} + \text{ROOH} \rightarrow \text{compound II} + \text{ROO}^{\cdot} \quad (7)$$

As the redox potential for the system $RO_2^{\cdot}/ROOH$ probably exceeds that of the system ROOH/ROH it is difficult to accept equation (7). Furthermore, as shown by Keilin & Nicholls (1958), no known donor reduces catalase compound I to II while being inactive towards peroxidase compound I. The decrease in the second-order velocity constant for the reaction with increasing peroxide concentration (Chance, 1949*b*) indicates that the effect may be catalytic, following an equation similar to (5) above. In this case, $K_m$ for ethyl hydroperoxide, acting as a ligand and not as a donor, would be about $2 \times 10^{-2}$ M.

The reaction of formate and catalase is of interest in the analysis of the reaction mechanism. Chance (1952*a*) measured the rates of reaction for both free catalase and compound I and commented on their similarity. If the progressive inhibitions of catalase by peroxide in the presence of acetate and of formate are also compared (Bonnichsen *et al.* 1947), it can be seen that formate fails to accelerate this inhibition. Evidently the reaction mechanisms of compound I with formate as donor and acetate as inhibitor must be very similar. For if the reaction of anions as inhibitors with compound I was fundamentally different from the corresponding reaction of two-electron donors, then formate, despite its donor action, would, in the presence of an excess of peroxide, be as effective an inhibitor as acetate. Formate thus has a threefold action on catalase: (i) as a ligand for the free enzyme, (ii) as a two-electron donor for compound I, and (iii) as a catalyst for compound II decomposition. And at least part of the mechanism seems to be very similar in all three cases.

Another peculiarity in this set of reactions, the very low affinity of catalase compound II for cyanide (Table 6), can perhaps be correlated with the fact that in the binding of cyanide by free enzyme the bond type changes from ionic to covalent, and the absence of observable spectral change in compound II indicates that the bonding remains covalent with and without cyanide.

The variation in maximal velocity constant (Fig. 5 and Table 5) may well be due to differences between enzyme samples; it is possible that this constant takes on different values for different ligands, but the observed variation is not very large. The absence of catalytic effect of anions on one-electron reactions with added donors indicates that the endogenous donor reaction is fundamentally different from that with exogenous donors. The reducing equivalents involved are associated with the same enzyme molecule as the peroxide compound decomposing (Table 1), and their 'activity' is altered not by oxidation but by processes altering the protein configuration.

One interpretation of this is that the electrons required are introduced from the protein via the haem-linked group, the protein acting as an electron conductor (Evans & Gergely, 1949). The weak reducing power of such electrons may be offset by the powerful oxidizing capacity of the peroxide compounds.

Fig. 6 illustrates the reactions postulated in explanation of the present results, and summarizes some of the kinetic data. The formal simplicity of this Figure conceals many difficulties of chemical interpretation. Not only is it necessary to suppose that the haem iron in the higher-valency states of compounds I and II is able to form complexes, but also that the dissociation constants for these complexes are closely similar to those formed by the iron in the ferric state (Table 5). If similar structures be postulated for the corresponding peroxidase compounds, Fig. 6 does not indicate any reason for the much lower affinity of those compounds for the ligands (Table 6). We have only the empirical correlation between the ability of the two compounds of type I to form complexes with anionic ligands, and their activity towards two-electron donors such as ethanol.

The evidence of the peroxide anion complexes is thus not compatible with the structures for the peroxide compounds themselves given previously either by Chance & Fergusson (1954) or by George (1953*a*), new chemical possibilities must be canvassed.

## SUMMARY

1. The influence of pH and anions on the formation and decomposition of catalase peroxide compound II was examined.

2. The rates of formation of compound II produced by ethyl hydroperoxide or the hydrogen peroxide generated in the notatin system, and of the decomposition induced by ethanol, show a similar proportionality to pH ($\delta \log_{10} k = -0{\cdot}6\ \delta$ pH).

3. The rates of reduction of compounds I and II by the endogenous donor concerned are first-order with respect to catalase concentration; they are not significantly affected by treating the enzyme with oxidizing agents, but are modified by processes which tend to denature the enzyme protein. It is concluded that the endogenous reductions are intramolecular reactions.

4. Anions accelerate both the reduction of compound I to II, and the reduction of compound II to free catalase, in the absence of added donors. The reactions show a Michaelis–Menten relationship with respect to anion concentration, from which a Michaelis constant ($K_m$) may be calculated.

5. For ionic ligands, $K_m$ values associated with the decomposition of compound II closely approximate the dissociation constants for the corresponding enzyme–anion complexes. For the covalent ligand cyanide, $K_m$ is much greater than the corresponding dissociation constant.

6. Azide reacts with compound II both catalytically and stoicheiometrically as a hydrogen donor.

7. At low hypophosphite concentrations, the rates of formation of the catalase–hypophosphite

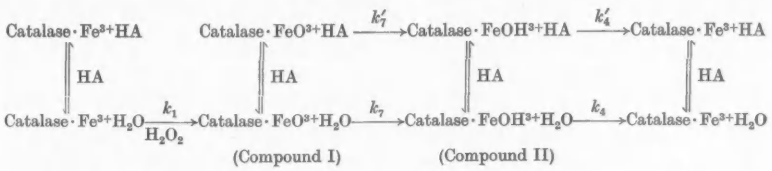


Fig. 6. Reactions of anionic ligands with catalase and its peroxide compounds. $k_7 = 1{\cdot}2 \times 10^{-2}$ sec.$^{-1}$; $k_4 = 9 \times 10^{-4}$ sec.$^{-1}$; $k'_7 = 0{\cdot}15$ sec.$^{-1}$ (corresponding to the experimentally obtained constant $k_{\text{I max.}}$); $k'_4 = 0{\cdot}08$ sec.$^{-1}$ (corresponding to the experimentally obtained constant $k_{\text{II max.}}$); HA represents an acid ligand forming an ionic complex with the free enzyme. All numerical values refer to pH 5, 20°.

complex from compound II and from free catalase are identical.

8. The anions have no observable effect on the reduction produced by added donors.

9. The actions of anions on catalase, peroxidase and metmyoglobin peroxide compounds are compared, and the relevance of the phenomena to the questions of the mode of action of catalase, and the nature of the endogenous donor, is discussed.

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# The Formation and Properties of Sulphmyoglobin and Sulphcatalase

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The preceding paper (Nicholls, 1961) dealt with the reactions of catalase peroxide compounds with various complex-forming ligands compared with the analogous reactions of peroxidase and metmyoglobin. Of covalent ligands, only cyanide was mentioned in that paper; the present paper describes the anomalous behaviour of another substance, hydrogen sulphide, which normally forms covalent complexes with haemoproteins.

The reactions of haemoglobin with hydrogen sulphide are complicated. At least two distinct derivatives occur: sulphaemoglobin and sulphide–methaemoglobin. The former is a green–purple (dichroic) compound produced when solutions of oxyhaemoglobin are allowed to stand in the presence of hydrogen sulphide. Sulphide–methaemoglobin is a red compound produced by the reversible reaction of methaemoglobin and hydrogen sulphide. This latter reaction is of the usual kind involving replacement of a water molecule at the sixth co-ordination site of the iron (Keilin, 1938), and further reactions can occur only if the sulphide is displaced. Sulphaemoglobin can, however, undergo reactions in its own right; thus it forms a complex with carbon monoxide, and its absorption band can be destroyed by ferricyanide (Nijveld, 1943). These reactions suggest that sulphaemoglobin is a ferrous derivative; the compound produced by ferricyanide would then be metsulphaemoglobin. No distinct spectral bands have been reported for

this compound, although Nijveld's figures suggest that acid and alkaline forms may occur.

The question of the reactants involved in the formation of sulphaemoglobin from oxyhaemoglobin has been the source of some controversy. Michel (1938) claimed that hydrogen peroxide is necessary, but Nijveld (1943) found that hydrogen peroxide gave rise to much choleglobin instead. Dalziel & O'Brien (1957) re-affirmed the need for peroxide, and showed the rapid formation of sulphcompounds in systems containing methaemoglobin peroxide; their systems, however, also contained dithionite, and several haemoglobin derivatives other than the peroxide compound, and their results do not therefore admit of one definitive interpretation. The chemical nature of sulphaemoglobin is also unknown. Keilin's (1938) study showed that both sulphaemoglobin and its ferric derivative differed from the normal reversible sulphide–methaemoglobin complex, i.e. its structure involved the modification of something other than the iron. Nijveld (1943) proposed an improbable structure with an allene carbon in the porphyrin ring. Lemberg & Legge (1949) suggested an even more improbable thiolhistidine structure; Foulkes, Lemberg & Purdom (1951) proposed replacement of the methene bridge $\geqslant C{\cdot}CH{=}C<$ by the grouping $\geqslant C{\cdot}CH(SH){\cdot}C(OH)<$ but offered no evidence in favour of this idea.

The action of hydrogen sulphide on the haem enzyme catalase has also been the subject of some disagreement. Zeile, Fawaz & Ellis (1940) reported the reduction of a preparation of liver catalase to the ferrous form under the action of hydrogen sulphide and dithionite. Keilin & Hartree (1945) could not repeat this observation although they had found a light-sensitive inhibition of catalase by carbon monoxide in the presence of thiols such as cysteine and glutathione (Keilin & Hartree, 1938). Beers & Sizer (1954) demonstrated that the pattern of inhibition of catalase by hydrogen sulphide is more complicated than that produced by other ligands. These facts suggest the occurrence of more than one reaction between catalase and hydrogen sulphide.

With peroxidase fewer peculiarities have been reported. There was a claim that thiols were inhibitory, but Randall (1946) showed that they act as hydrogen donors for the enzymic activity of peroxidase.

Thus it is known that some unusual interactions between hydrogen sulphide and haemoproteins can occur. But the nature of the intermediates formed and their chemical relationships with each other and with the parent haemoprotein are unknown. And it is also not known whether the phenomena observed in catalase are analogous in any way to those occurring with haemoglobin.

This paper will show that all these observations can be interpreted in terms of the chemistry of haemoprotein peroxide compounds, and that the reactions involved may throw light upon the nature of those compounds. The existence of sets of ferrous and ferric haem derivatives analogous to those produced with the parent protohaem enzymes will be demonstrated for the sulph-compounds.

## EXPERIMENTAL

The materials and methods used were as described in the preceding paper (Nicholls, 1961). Most of the observations were carried out with a Zeiss bench microspectroscope, and the major points checked quantitatively in a Hilger Uvispek spectrophotometer with a glass prism giving high dispersion in the visible region.

Sodium sulphide and ammonium sulphide (both AnalaR) were used as sources of hydrogen sulphide. The ammonium sulphide was preferred for spectrophotometric experiments, as the sodium sulphide contained small quantities of polysulphides which tended to precipitate at neutral pH.

The hydrogen sulphide content of neutralized solutions prepared from these sources was estimated by adding the sulphide solution to a known excess of iodine solution in dilute sulphuric acid. The iodine which remained was titrated against standard sodium thiosulphate, with starch as indicator at the end point.

## RESULTS

### *Formation and properties of sulphmyoglobin*

The addition of hydrogen sulphide in stoicheiometric quantities to a solution of metmyoglobin peroxide compound (approx. 30 $\mu$M) at pH 8 (0·05 M-borate–potassium chloride buffer) resulted in the rapid formation of a mixture of metsulphmyoglobin and small quantities of sulphmyoglobin, with the disappearance of the peroxide compound spectrum. [At the lowest sulphide concentrations employed ($\sim$10 $\mu$M, see Table 1), the reaction was complete within seconds, indicating a second-order velocity constant greater than 1 mM$^{-1}$ sec.$^{-1}$.] Sulphmyoglobin was recognized spectroscopically by its intense absorption band at 617 m$\mu$; metsulphmyoglobin was recognized by the bands at 595 and 715 m$\mu$. Excess of peroxide had been removed, after the formation of the metmyoglobin peroxide compound, by the addition of small amounts of catalase; this was necessary to prevent secondary reactions such as (4) below. When excess of sulphide was added, all the metsulphmyoglobin was reduced to the ferrous form in a secondary reaction.

If metmyoglobin peroxide is written as $MbFeO^{2+}$ (cf. ferryl ion or $FeO^{2+}$, George & Irvine, 1955), the reaction can be given as

$$MbFeO^{2+} + H_2S \rightarrow \begin{cases} MbSFe^{2+} + H_2O & (a) \\ MbSFe^{3+} + H_2O + e & (b) \end{cases} \quad (1)$$

The $MbSFe^{3+}$ in reaction (*b*) can be reduced by a second molecule of $H_2S$

$$MbSFe^{3+} + H_2S \rightarrow MbSFe^{2+} + H^+ + SH^{\cdot} \quad (2)$$

The major overall reaction is then:

$$MbFeO^{2+} + 2H_2S \rightarrow MbSFe^{2+} + H_2O + [H^{\cdot}] + [SH^{\cdot}] \quad (3)$$

(The fate of the extra reducing equivalent, designated by $H^{\cdot}$, is unknown.) Table 1 gives the results of a spectrophotometric experiment to demonstrate the stoicheiometry of the reaction. Table 2 gives some of the spectroscopic data obtained for the ferrous sulphmyoglobin compared with ordinary ferrous myoglobin, and shows the remarkable intensity of the absorption band in the red, which is comparable with that of the α-band of a haemochromogen.

Like sulphaemoglobin (Nijveld, 1943), sulphmyoglobin combines with carbon monoxide (bubbled through the cuvette) but not with oxygen. As Table 2 shows, the extinction coefficients of the Soret bands of the sulph-compounds are less than those of the normal derivatives. The carbon monoxide derivative of sulphmyoglobin in particular has a rather low band compared with carbon monoxide–myoglobin; but a millimolar extinction coefficient of 105 does not justify Lemberg & Legge's (1949) statement that 'carboxysulphaemoglobin has only a weak absorption (in the Soret region)'.

Like other ferrous haem compounds, sulphmyoglobin fails to react with anionic ligands (fluoride, azide, sulphide) to form complexes. It does, however, undergo a very slow reaction with sodium azide, in which the product is reduced myoglobin. This reaction requires high azide concentrations ($\geqslant 0{\cdot}1$ M), but is not due to the change in ionic strength, since chloride produces no such effect. The azide apparently reacts with the haem to remove the modifying grouping.

Oxidizing agents, such as ferricyanide, ethyl hydroperoxide and molecular oxygen, convert sulphmyoglobin into the ferric form, metsulphmyoglobin. That this change involves only one electron was shown for haemoglobin by Nijveld (1943). Hydrogen peroxide, however, unlike the other oxidizing agents, destroys the sulphmyoglobin spectrum and produces oxymyoglobin. Table 3 gives the results of a typical experiment of this nature, with the calculated concentrations of

Table 1. *Titration of metmyoglobin peroxide with hydrogen sulphide*

60 μM-Metmyoglobin in 2·6 ml. of borate buffer, pH 8 (0·156 μmole of total metmyoglobin); peroxide compound produced by addition of 1 μmole of ethyl hydroperoxide; sulphide added as ammonium sulphide. 20°. Changes of *E* were measured at 580 mμ, millimolar extinction coefficients of 8·1 for the peroxide compound and 2·5 for metmyoglobin at this wavelength being used (cf. Keilin & Hartree, 1955; George & Irvine, 1952).

| | $H_2S$ added (μmole) | Metmyoglobin peroxide destroyed (μmole) | Haem/$H_2S$ ratio |
|---|---|---|---|
| 1 | 0·032 | 0·023 | 0·72 |
| 2 | 0·08 | 0·077 | 0·96 |
| 3 | 0·032 | 0·029 | 0·91 |
| Total | 0·144 | 0·129 | 0·90 |

(Further addition of 1 μmole of $H_2S$ reduced the metsulphmyoglobin to sulphmyoglobin.)

Table 2. *Absorption bands of myoglobin and sulphmyoglobin*

Extinction coefficients for myoglobin were obtained experimentally, for reference purposes the value for the α-band of myoglobin itself given by Keilin & Hartree (1955) being used; values for sulphmyoglobin were obtained by adding excess of sulphide to solutions containing known amounts of metmyoglobin peroxide and assuming 100% conversion (cf. the titration data of Table 1).

| Compound | α-Band λ (mμ) | α-Band $10^{-3}\epsilon$ | Soret band λ (mμ) | Soret band $10^{-3}\epsilon$ |
|---|---|---|---|---|
| Myoglobin | 558 | 11·0 | 433 | 110 |
| Carboxymyoglobin | 576 | 12·0 | 422 | 160 |
| Sulphmyoglobin | 617 | 17·5 | 420 | 90 |
| Carboxysulphmyoglobin | 613 | 19·0 | 422 | 105 |

Table 3. *Action of hydrogen peroxide on sulphmyoglobin*

Borate buffer, pH 8·5; 20°; sulphmyoglobin formed by addition of 0·3 mM-$H_2S$ (as ammonium sulphide) to 85 μM-metmyoglobin peroxide. Extinction coefficients for sulphmyoglobin (SMb) were taken from Table 2 and Fig. 1; millimolar extinction coefficient of oxymyoglobin ($MbO_2$) at 580 mμ is taken as 13.

| Conditions | *E* 618 mμ | *E* 580 mμ | $10^5 \times$ Calculated concn. (M) SMb | $10^5 \times$ Calculated concn. (M) $MbO_2$ |
|---|---|---|---|---|
| (1) Sulphmyoglobin as formed | 1·18 | 0·54 | 7·5 | 0·9 |
| (2) Sulphmyoglobin with 1 mM-$H_2O_2$ | 0·50 | 0·61 | 2·5 | 3·8 |

sulphmyoglobin and oxymyoglobin before and after the addition of hydrogen peroxide. This back reaction is responsible for the variability in yield of sulphmyoglobin when excess of hydrogen peroxide is present.

As this reaction does not occur with ethyl hydroperoxide, it must involve the reducing action of hydrogen peroxide, and may be formulated

$$MbSFe^{2+} + H_2O_2 \rightarrow MbFe^{2+}O_2\ (+H_2S?) \qquad (4)$$

We therefore have two ways of re-forming myoglobin from ferrous sulphmyoglobin: (*a*) by a reaction with sodium azide; (*b*) by a reaction with hydrogen peroxide.

The metsulphmyoglobin, formed either directly (equation 1) or by oxidation of sulphmyoglobin (above), was found to have properties which clearly established the independence of the haem modification concerned from the iron atom. First, it is reversibly oxidized and may be reduced back to sulphmyoglobin by agents such as dithionite and hydrogen sulphide itself. Secondly, it forms a series of complexes with anions, of which Nijveld (1943) mentions the one with cyanide.

Complex-formation occurs with cyanide, azide and fluoride added in approximately 10 mM concentration at pH 8·0 (but not with sulphide, which acts as reductant). An ionization occurs between pH 8·5 and 9·0, giving an alkaline form. Hydrogen peroxide gives rise to a peroxide compound. The spectra of all these derivatives show analogies to those of the corresponding compounds of normal metmyoglobin, but the absorption bands (other than the Soret band) are displaced far into the red end of the spectrum (Fig. 2).

With the cyanide derivative, the relative intensities of the bands at 590 and 550 m$\mu$ were found to be variable, which is only comprehensible if these two bands belong to different compounds, the former to the true cyanmetsulphmyoglobin, the latter to cyanmetmyoglobin.

In fact, cyanide regenerates metmyoglobin from metsulphmyoglobin, and complete reversal can be achieved by adding dithionite to the mixture. In the latter reaction the spectrum of the unstable cyanmyoglobin as an intermediate could be observed, but no absorption bands attributable to a cyansulphmyoglobin complex were seen.

$$\left.\begin{aligned} &MbSFe^{3+}CN + HCN \rightleftharpoons MbFe^{3+}CN \\ &\qquad\qquad (+HSCN?) \quad (a) \\ &MbFe^{3+}CN \xrightarrow{Na_2S_2O_4} MbFe^{2+}CN \rightarrow MbFe^{2+} \quad (b) \end{aligned}\right\} (5)$$

This represents a third way of re-forming myoglobin from sulphmyoglobin.

The metsulphmyoglobin peroxide compound behaves in a similar fashion to metmyoglobin peroxide (George & Irvine, 1955). It is produced rapidly by hydrogen or alkyl hydrogen peroxides, although even at pH 8·0 it is unstable compared with the normal metmyoglobin peroxide compound. The velocity constants for its decomposition by ferrocyanide or by *p*-cresol were similar to those for the metmyoglobin peroxide compound.

Fig. 1 shows the absorption spectra, in the visible and Soret regions, of sulphmyoglobin and metsulphmyoglobin compared with their normal counterparts. Fig. 2 shows the visible spectra of some of the derivatives of metsulphmyoglobin, and demonstrates the analogy with and differences from the same derivatives of metmyoglobin. The nature of the compounds responsible for these spectra, their mode of formation and their properties, which may throw light upon the problem of the nature of the haemoprotein peroxide compounds, are discussed later on, after the description of the corresponding catalase compounds.

*Formation and properties of sulphcatalase*

The behaviour of catalase towards hydrogen sulphide is in many ways analogous to that of metmyoglobin, and this behaviour can account for many of the peculiarities of catalase in the presence of thiols.

In those systems in which cysteine was found to accelerate the decomposition of compound II (the secondary peroxide compound of catalase, in which the haem iron is effectively in a quadrivalent oxidation state), the transitory formation of a compound with an absorption band at 635 m$\mu$ could be observed (Keilin & Nicholls, 1958*a*). The following evidence indicates that this effect was due to hydrogen sulphide, which was present as an impurity in the cysteine and reacted with compound II in a manner analogous to its reaction with metmyoglobin peroxide.

Hydrogen sulphide reacts rapidly with compound II of catalase to give a derivative with an intense absorption band at 635 m$\mu$. This reaction is illustrated by Fig. 3, which indicates its main features, namely: (*a*) rapid decrease in absorption at 565 m$\mu$; (*b*) concomitant increase in absorption at 635 m$\mu$; (*c*) slow subsequent decrease in the 635 m$\mu$ band.

Fig. 4 shows the major changes in the characteristics of the final spectrum, and demonstrates the further important features of the overall reaction:

(i) The final disappearance of the 635 m$\mu$ band is in at least two phases and there is no common isosbestic point.

(ii) The 635 m$\mu$ compound reacts with carbon monoxide (bubbled through the spectrophotometer cuvette) and is thereby stabilized against its subsequent disappearance.

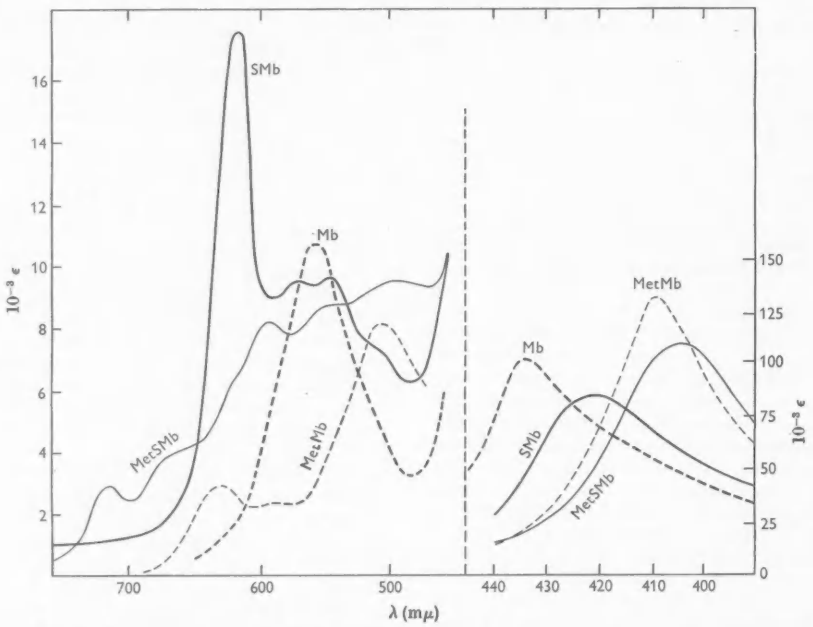



Fig. 1. Visible and Soret spectra of sulphmyoglobin and metsulphmyoglobin compared with normal myoglobin and metmyoglobin. Myoglobin and metmyoglobin in phosphate buffer, pH 6·0. Sulphmyoglobin and metsulphmyoglobin in borate buffer, pH 8·0. Mb, Myoglobin; MetMb, metmyoglobin; SMb, sulphmyoglobin; MetSMb, metsulphmyoglobin. Absolute extinction coefficients were calculated from measurements on 40 $\mu$M-myoglobin solutions in 1 cm. glass cuvettes at 20°.

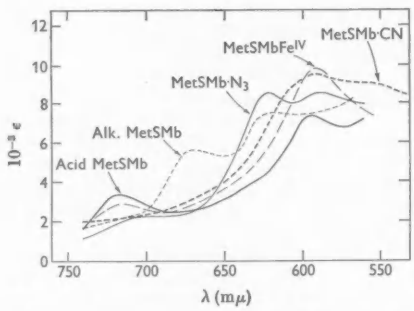



Fig. 2. Visible absorption spectra of metsulphmyoglobin and some of its derivatives. Alkaline metsulphmyoglobin (Alk. MetSMb) in borate buffer, pH 9·4. Other derivatives: acid (Acid MetSMb), azide (MetSMb $N_3$), cyanide (MetSMb CN), peroxide (MetSMbFe$^{IV}$), in borate buffer, pH 8·0. Absolute extinction coefficients were calculated from measurements on 40 $\mu$M-metsulphmyoglobin solutions in 1 cm. glass cuvettes at 20°.

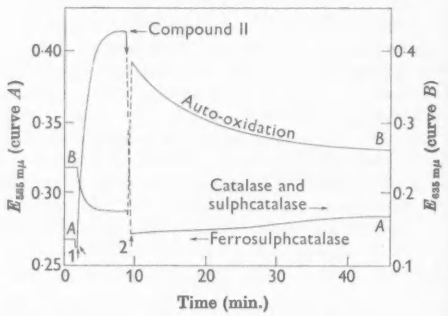



Fig. 3. Effect of hydrogen sulphide on catalase peroxide compound II. 20 $\mu$M-Catalase (haem concentration) in phosphate buffer, pH 6, at 20°; compound II was formed by addition of ethyl hydroperoxide (1 $\mu$mole) as indicated at arrow 1, and destroyed by addition of sodium sulphide (5 $\mu$moles) at arrow 2. Curve *A*: measurements at 565 m$\mu$ (left-hand ordinate); curve *B*: measurements at 635 m$\mu$ (right-hand ordinate).

The above empirical facts are interpreted in terms of reactions of the following type:

$$\text{Catalase compound II} + H_2S \rightarrow \text{catalaseS}\cdot Fe^{3+} + H_2O \quad (6)$$

$$\text{CatalaseS}\cdot Fe^{3+} \xrightarrow{O_2} \text{catalaseS}\cdot Fe^{3+} \quad (7)$$

$$\text{CatalaseS}\cdot Fe^{3+} \xrightarrow{O_2 \text{ (or } H_2O_2)} \text{catalase}\cdot Fe^{3+} (+\text{oxidized S?}) \quad (8)$$

Reaction (6) is faster than that with metmyoglobin peroxide, equation (1); unlike the last-mentioned reaction, the resulting compound (ferrosulphcatalase) is entirely ferrous, there being no analogues of (1) *b*. Table 5 shows that the stoicheiometry of reaction (6) is the same as that with metmyoglobin.

Reaction (7) indicates the auto-oxidation of the ferrosulphcatalase. Fig. 3 shows this to be a reaction first-order with respect to ferrosulphcatalase, with a velocity constant of about $2 \times 10^{-3}$ sec.$^{-1}$ ($10\,M^{-1}$ sec.$^{-1}$ for dissolved oxygen concentration of 0·2 mM). This oxidation is not promoted by potassium ferricyanide, nor is ferrosulphcatalase re-formed by the addition of dithionite or excess of sulphide; this behaviour is similar to that of normal catalase (Keilin & Hartree, 1936).

Reaction (8), indicated by the gradual appearance of an absorption band at 622 mμ (Fig. 4) due to free catalase, is discussed in the section on 'reversibility' below.

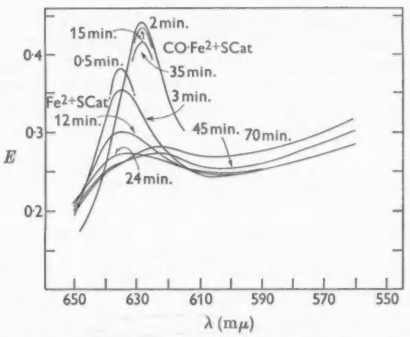



Fig. 4. Decomposition of ferrosulphcatalase in air, in the presence and the absence of carbon monoxide. 17 μM-Catalase compound II in phosphate buffer, pH 6·1, at 20° under aerobic conditions; 5 μmoles of sodium sulphide were added at zero time. $Fe^{2+}$SCat: Ferrosulphcatalase; CO·$Fe^{2+}$SCat: carboxyferrosulphcatalase. Spectra for product at 0·5, 3, 12, 24, 45, 70 min. are given, together with the corresponding spectra for solutions initially saturated with carbon monoxide, at 2, 15 and 35 min.

Table 5 shows the spectroscopic data obtained for catalase derivatives in the visible region of the spectrum. Sulphcatalase forms complexes with cyanide, sulphide, fluoride and azide. Sodium dithionite does not reduce it to ferrosulphcatalase, but does have some effect on the attached sulphur (see below). The catalatic activity of residual free catalase prevents observation of the action of hydrogen peroxide; but ethyl hydroperoxide gives an intermediate with a spectrum identical with that of compound II (bands at 540 and 570 mμ), and this intermediate produces free catalase on decomposition. Table 6 gives the positions of the absorption bands of the ferric derivatives, which may be compared with those of metsulphmyoglobin in Table 7.

The sulphide complex, sulphide–sulphcatalase, is an inhibited catalase derivative in which the same inhibitor, hydrogen sulphide, has acted in two ways upon the same haem prosthetic group. One molecule of the sulphide has formed a thermodynamically irreversible compound with the porphyrin ring, while a second has produced a normal reversible complex with the central iron atom.

### *Reversibility of sulphcatalase formation*

The light-reversible inhibition of catalase by carbon monoxide obtained by Keilin & Hartree (1938) in the presence of thiols such as cysteine, and the partial reversibility of the sulphide inhibition (Beers & Sizer, 1954), indicates the possibility of the regeneration of free catalase from sulphcatalase.

Table 4. *Titration of catalase compound II with hydrogen sulphide*

20 μM-Liver catalase; 0·08 M-phosphate buffer, pH 5·0; 20°; peroxide compound formed by addition of 1 μmole of ethyl hydroperoxide; sulphide was added as ammonium sulphide. Final volume: 2·5 ml. Changes of $E$ were measured at 565 mμ and 635 mμ. Assuming: $\Delta E$ at 565 mμ of 0·025 $\equiv 10^{-2}$ μmole of haem (compound II). $\Delta E$ at 635 mμ of 0·036 $\equiv 10^{-2}$ μmole of haem (ferrosulphcatalase).

| | $H_2S$ added (μmole) | Compound II titrated (μmole) | Ferrosulphcatalase formed (μmole) | Haem/$H_2S$ ratio |
|---|---|---|---|---|
| 1 (*a*) | 0·016 | 0·012 | 0·021 | 0·69 |
| 1 (*b*) | 0·016 | 0·011 | | |
| 2 | 0·032 | 0·022 | 0·018 | 0·62 |
| 3 | 0·031 | 0·028 | 0·029 | 0·92 |

As with metmyoglobin, some ways of reversing the formation of the sulph-compound do exist. In fact, the substituent in sulphcatalase is more labile than that of sulphmyoglobin. Catalase is regenerated under the following conditions:

(*a*) As indicated in Fig. 4, and expressed by equation (7), the substituent group is labile to oxidizing agents, including oxygen, ethyl hydroperoxide and possibly hydrogen peroxide, when the iron is in the ferric state. Sulphcatalase therefore differs from metsulphmyoglobin in that it does not form a stable peroxide derivative.

$$\text{CatalaseS}\cdot Fe^{3+} \xrightarrow{\text{oxidants}} \text{catalase}\cdot Fe^{3+} \quad (+\text{oxidized sulphur?}) \qquad (9)$$

(or derivative, e.g. compound II)

(*b*) The ligands cyanide, azide and fluoride form stable compounds with sulphcatalase but they accelerate the regeneration of free ferric catalase from ferrosulphcatalase (cf. the production of myoglobin from sulphmyoglobin in the presence of azide or cyanide). The transition is not promoted by all ligands for ferric catalase, as in this respect formate is inactive. Carbon monoxide inhibits the reaction by combining with ferrosulphcatalase.

$$\text{CatalaseS}\cdot Fe^{2+} \xrightarrow[+\text{oxidant?}]{F^-,\ CN^-\text{ or }N_3^-} \text{catalase}\cdot Fe^{3+} \qquad (10)$$

(or derivative, e.g. catalase–anion complex)

(experiments carried out at pH 5, 20°, with 10 mM-anion).

Table 5. *Spectroscopic data for ferrosulphcatalase*

The sulph-compounds were prepared by adding excess of sulphide to a solution containing a known concentration of compound II, and the extinction coefficients were calculated assuming 100% conversion.

| | α-Band | |
|---|---|---|
| Compound | $\lambda_{max.}$ (mμ) | $10^{-3}\epsilon$ |
| Catalase* | 622 | 10·5 |
| Catalase compound II | 568 | 20·0* |
| Ferrosulphcatalase | 635 | 21·0 |
| Carboxyferrosulphcatalase | 627 | 24·0 |

* Liver catalase; pH 5; 20°.

(*c*) Sodium dithionite reacts with sulphcatalase and ferrosulphcatalase; in the former case ferric catalase is produced, in the latter a mixture of ferric and ferrous catalase. Both reactions seem to involve the destruction of a certain amount of catalase.

$$\left.\begin{array}{l} \text{CatalaseS}\cdot Fe^{3+} \xrightarrow{Na_2S_2O_4} \text{catalase}\cdot Fe^{3+} \quad (+\text{reduced S?}) \\ \qquad\qquad\qquad\qquad\uparrow \\ \qquad\qquad\qquad\qquad O_2\text{ (rapid)} \\ \qquad\qquad\qquad\qquad\downarrow \\ \text{CatalaseS}\cdot Fe^{2+} \xrightarrow{Na_2S_2O_4} \text{catalase}\cdot Fe^{2+} \quad (+\text{reduced S?}) \end{array}\right\} \qquad (11)$$

(experiment carried out at pH 6, 20°, a few milligrams of sodium dithionite/ml. being used). The formation of ferrous catalase in the second reaction is indicated by the disappearance of the band at 635 mμ without the production of the 620 mμ band, but a broad absorption in the green; the latter may be converted into two bands at 545 and 575 mμ by passing carbon monoxide into the mixture. This phenomenon probably accounts for the claim of Zeile *et al.* (1940) to have obtained reduced catalase by the action of hydrogen sulphide followed by dithionite.

*Reactions of sulphide with other peroxide compounds*

Of the five known haemoprotein peroxide compounds which can be reduced by hydrogen donors (catalase and peroxidase compounds I and II, metmyoglobin peroxide), only metmyoglobin peroxide and catalase peroxide compound II seem to react with hydrogen sulphide to give sulph-compounds.

Catalase compound I, which might be expected to give rise to ferric sulphcatalase directly, does not appear to do so. When hydrogen sulphide was added to catalase solutions and ethyl hydroperoxide added subsequently, the first observable derivative was ferrosulphcatalase. Either the transition of compound I to II must be faster than the reaction of compound I with hydrogen sulphide, or the latter reaction must be such as not to

Table 6. *Spectroscopic data for sulphcatalase derivatives*

| | Position of visible absorption bands (mμ) | |
|---|---|---|
| Derivative | Catalase | Sulphcatalase |
| Acid form ($H_2O$) | 622, 535, 500 | 710, (635)*, 585 |
| Alkaline form ($OH^-$) | Unknown | Unknown |
| Cyanide | (585), 555 | 645 |
| Fluoride | 595 | 680 |
| Azide | 620 | ~700 |
| Sulphide | (640)*, 585, 550 | 645 |
| Ethyl hydroperoxide | 568, 535 | (568, 535)† |

* Possibly due to sulphide–sulphcatalase contamination. † Apparently identical with normal compound II.

Table 7. *Spectroscopic data for metsulphmyoglobin derivatives*

| Derivative | Position of visible absorption bands (mμ): Metmyoglobin | Metsulphmyoglobin |
|---|---|---|
| Acid form ($H_2O$) | 635, 505 | 715, 595 |
| Alkaline form ($OH^-$) | 590, 540 | 670, 622 |
| p*K* | 8·9 | Between 8·5 and 9·0 |
| Cyanide | (580), 545 | 590, 550* |
| Fluoride | 610 | 680 |
| Azide | 570, 545 | 622, 590 |
| Hydrogen peroxide | 580, 550 | 590 |
| Sulphide | 585, 545 | Does not exist |

* Probably due to CN–metmyoglobin re-formed from the sulphmyoglobin by action of cyanide (see text).

involve the production of an inhibitory sulph-compound.

The addition of ammonium sulphide to solutions of peroxidase compound I and II caused the disappearance of the absorption spectra of the peroxide compounds and the reappearance of the band of free peroxidase at 640 mμ. Peroxidase appears to act as oxidant towards sulphide as hydrogen donor:

$$\text{Peroxidase compound II} + H_2S \rightarrow \text{peroxidase}\cdot Fe^{3+} + SH^{\cdot} \quad (12)$$

*Reactions with complex thiols and with selenide*

The reaction in which sulph-compounds are formed is only produced by hydrogen sulphide. As shown by Keilin & Nicholls (1958*a*), complex thiols such as cysteine do not bring it about. Furthermore, an analogous reaction does not occur with hydrogen selenide. The generation of hydrogen selenide from aluminium selenide in a solution of metmyoglobin peroxide resulted in the formation of oxymyoglobin, and eventually reduced myoglobin, owing to the removal of oxygen by auto-oxidation.

$$MbFeO^{2+} + H_2Se \rightarrow MbFe^{2+}H_2O + Se \quad (13)$$

No bands attributable to a 'selenomyoglobin' were observed.

## DISCUSSION

The above results bear upon two theoretical issues: (*a*) the nature and properties of sulph-compounds as a general problem in haem chemistry, and (*b*) the structures and properties of the normal haemoprotein peroxide compounds.

Michel (1938) formulated the sulphaemoglobin-producing reaction as:

$$Hb + H_2S + H_2O_2 \rightarrow HbS + 2H_2O \quad (14)$$

Nijveld (1943), however, thought that oxyhaemoglobin was directly involved, thus:

$$HbO_2 + H_2S(Hb) \rightarrow (Hb\text{–}2H)S + 2H_2O + Hb \quad (15)$$

According to the results given above, equation (14) states the reactants and oxidation state of sulphmyoglobin correctly (apart from the oxidation state of initial haem) whereas equation (15) does not. The production of large amounts of choleglobin in Nijveld's experiments can be attributed to the presence of reducing agents such as ascorbic acid (Lemberg, Legge & Lockwood, 1941). But it cannot be stated that a reaction involving oxyhaemoglobin does not occur, because equation (4) may represent a reversible system; thus the sulphmyoglobin in Table 3 is not completely destroyed by peroxide. Nijveld's view that sulphaemoglobin may be reconverted into haemoglobin is supported by the present data for myoglobin; azide and hydrogen peroxide can now be added to cyanide as reagents bringing this about.

In the absence of such reagents the sulph-compounds remain quite stable and the various derivatives here described may provide a new way of investigating the relations between porphyrin, iron and protein in the reactions promoted by haemoproteins. Thus it would be interesting to study the equilibria between metsulphmyoglobin and ligands more quantitatively and compare the results with those for metmyoglobin. Nijveld, unaware of these possibilities, did attempt to measure the redox potential of the sulphaemoglobin/metsulphaemoglobin system, obtaining a value of +0·12 to +0·14 v at pH 7. Preliminary experiments in this laboratory, in which the haemoproteins were equilibrated under nitrogen with the ferric–ferrous oxalate system (Michaelis & Friedheim, 1931), indicate a potential of +0·13 v for the sulphmyoglobin–metsulphmyoglobin system, compared with +0·04 v for myoglobin–metmyoglobin (pH 6·5, 0·024 M-phosphate, 20°). This may help to explain the greater reducibility of metsulphmyoglobin by hydrogen sulphide, and suggests that the strange spectrum of the reduced form may be associated with some stereochemical or electronic stabilization.

The absence of an oxygenated form of sulphmyoglobin suggests a comparison between the formation of carbon monoxide complexes in the protohaem and sulphaem derivatives. The kinetics of sulphmyoglobin auto-oxidation might give other information on the formation of oxygen complexes than that available by direct spectroscopic examination.

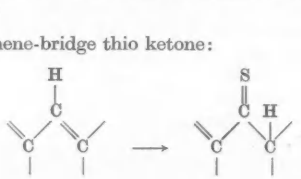
The reactions between sulphmyoglobin and azide or cyanide show that considerable caution must be employed in concluding from an effect of such agents that a ferric haem compound is

involved. This becomes even more evident in the behaviour of catalase where fluoride also reacts in some way with the ferrous sulph-compound (equation 10 above). The latter case is somewhat reminiscent of the effect of anions on catalase peroxide compounds (Nicholls, 1961), but its kinetics have not yet been examined.

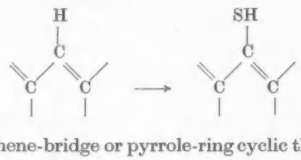
For catalase, the results explain the disagreement about the reduction of catalase in the presence of hydrogen sulphide, and suggest the identity of the anomalous 640 m$\mu$ band reported for sulphide–catalase complex (Zeile & Hellstrom, 1930; Keilin & Hartree, 1936) with that of sulphide–sulphcatalase. And, incidentally, they provide a new method of reducing the iron of catalase to the ferrous state. Free catalase is inert towards dithionite unless partially denatured (freeze-dried catalase). Compound I can be reduced to nitric oxide ferrocatalase by azide, a comparatively weak reductant (cf. Keilin & Hartree, 1945). Likewise, the weak reducing agent hydrogen sulphide is now shown to reduce compound II to ferrosulphcatalase. Conversely, metmyoglobin is readily reduced by dithionite but its peroxide compound is not so susceptible to reduction by azide or sulphide. The reversibility of the sulphcatalase formation permits an analysis of the effects reported by Keilin & Hartree (1938) and by Beers & Sizer (1954). Unlike the carbon monoxide inhibition of the catalatic reaction in the presence of azide, the inhibition produced in the presence of thiols is only slowly reversed by light (a lag phase of about 1 min. is shown in Keilin & Hartree's 1938 paper). In Beers & Sizer's experiments, sulphide was the only inhibitor whose effect decreased in some cases after the addition of peroxide (indicating a reversal of the inhibition by the oxidant).

Although therefore many problems in kinetic behaviour can be interpreted formally by reference to the sulph-compounds, exact chemical formulation of most of the reactions is not possible. This is largely because the chemical structure of the sulph-compounds themselves is unknown. Some conclusions may, however, be drawn. Thus the molecular structure indicated seems to be the same in sulphmyoglobin and sulphcatalase, each containing one more sulphur atom than the normal haemoprotein. The sulphur cannot be bound to the iron atom, as ligands combine with the latter, both in ferric and ferrous forms. Possibilities include:

(1) Methene-bridge thio ketone:



(2) Methene-bridge thiol:



(3) Methene-bridge or pyrrole-ring cyclic thio ether:



The comparatively strong Soret bands support the thiol structure (conjugate double bonds remain), but the absence of effect of thiol reagents (lead acetate and iodoacetate) supports structures 1 or 3. The thio ketone form (1) is perhaps the most likely.

In any case it is difficult to account for the spectra. Tables 6 and 7 show that for metsulphmyoglobin the absorption bands of ionic complexes are shifted 80 m$\mu$, those of covalent complexes about 40 m$\mu$ towards the infrared, whereas for sulphcatalase the bands of all complexes are moved some 90 m$\mu$ towards the infrared. The bands of the ferrous compounds (Tables 2 and 5) are shifted to a similar extent, but the spectral pattern is changed.

Some information about the secondary peroxide compounds is obtained from their reactions with hydrogen sulphide. There are three types of reaction:

$$\text{Haem}\cdot\text{FeO}^{2+} + \text{H}_2\text{S} \rightarrow \begin{cases} \text{HaemS}\cdot\text{Fe}^{2+} + \text{H}_2\text{O} & (a) \\ \text{HaemS}\cdot\text{Fe}^{3+} + \text{OH}^- + [\text{H}^\cdot] & (b) \\ \text{Haem}\cdot\text{Fe}^{3+} + \text{OH}^- + [\text{SH}^\cdot] & (c) \end{cases} \qquad (16)$$

($a$) and ($b$) metmyoglobin reaction; ($a$) catalase reaction; ($c$) peroxide reaction.

The similar behaviour of metmyoglobin peroxide and catalase compound II is evidence of some chemical similarity, despite the spectroscopic differences. Indeed, unlike the situation described for the reactions with donors (Keilin & Nicholls, 1958$b$) and anions (Nicholls, 1961), there appears here to be a closer relation between catalase compound II and metmyoglobin peroxide than between catalase compound II and peroxidase compound II.

However, there is also an important difference. Myoglobin, which forms the $Fe^{4+}$ oxidation state directly from $Fe^{3+}$, forms a peroxide compound ($Fe^{4+}$) from the ferric sulph-derivative. Catalase, which forms the $Fe^{4+}$ state (compound II) via a $Fe^{5+}$ state (compound I), forms no peroxide compounds in the sulphcatalase form. This raises the question whether compound I involves interaction with the porphyrin ring in the region modified by

addition of the sulphur. This possibility will be discussed in a later paper.

The derivatives described in this paper are mainly of interest for the light they may throw upon haemoprotein chemistry in general. Similar compounds may, however, sometimes occur naturally. Sulphaemoglobin is found *in vivo* after some kinds of drug therapy, in cases of septicaemia and in poisoning with 2:4:6-trinitrotoluene (Lemberg & Legge, 1949). According to Lemberg & Legge, the hydrogen sulphide always arises from intestinal bacterial activity.

Sulphcatalase has not previously been reported but observations of inhibition by carbon monoxide in crude extract of liver catalase (Califano, 1934) may have been due to the formation of ferrosulphcatalase in such preparations.

The most interesting possibility is represented by myeloperoxidase. Its absorption spectrum, derivatives and reported behaviour show a close analogy with sulphmyoglobin and sulphcatalase (Agner, 1941). Agner (1958) has now crystallized this enzyme, and more evidence should soon be forthcoming.

## SUMMARY

1. Sulphmyoglobin is formed stoicheiometrically by the reaction of hydrogen sulphide and metmyoglobin peroxide. It combines with carbon monoxide, but not with oxygen, and is oxidized to metsulphmyoglobin by ferricyanide.

2. Metsulphmyoglobin behaves like metmyoglobin. Its iron atom is co-ordinated with a water molecule dissociating under alkaline conditions; it forms fluoride, cyanide and azide complexes, and a metsulphmyoglobin peroxide compound.

3. Myoglobin is re-formed from sulphmyoglobin by the action of azide or hydrogen peroxide; metsulphmyoglobin is slowly converted into metmyoglobin in the presence of cyanide.

4. Ferrosulphcatalase is formed by the action of hydrogen sulphide on catalase compound II. It combines with carbon monoxide and is oxidized to (ferric) sulphcatalase by oxygen but not by ferricyanide.

5. Sulphcatalase forms complexes with fluoride, cyanide, sulphide and azide; it is not reducible by dithionite, does not give an alkaline form and does not produce peroxide compounds. In the sulphide–sulphcatalase complex, the sulphide is inhibiting the same haem group in two ways: by irreversibly modifying the porphyrin and by forming a reversible complex with the iron.

6. Catalase is regenerated from sulphcatalase by oxidizing agents such as oxygen and alkyl peroxides; its aerobic formation from ferrosulphcatalase is accelerated by cyanide, azide and fluoride.

7. Sulph-compounds cannot be produced from any peroxidase peroxide compounds, nor from catalase compounds I or III. Hydrogen selenide does not have an analogous effect to that of sulphide.

8. The results are discussed with reference to the problems of the chemical nature of both sulphcompounds and peroxide compounds and the possibility of the natural occurrence of sulphcompounds.

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# Methods for the Quantitative Estimation of *N*-Acetylneuraminic Acid and their Application to Hydrolysates of Sialomucoids

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The sialic acids (Blix, Gottschalk & Klenk, 1957) are widely distributed components of many sialomucoids (Blix, 1940; Zilliken & Whitehouse, 1958). They are readily obtained from these complexes by mild acid hydrolysis and more specifically by hydrolysis with neuraminidase, an enzyme found in many bacterial filtrates and the myxo group of viruses (Hirst, 1942; Gottschalk, 1958*a*, *b*).

Several techniques have been developed for the determination of the sialic acids, e.g. tryptophan and perchloric acid (Seibert, Pfaff & Seibert, 1948), diphenylamine (Hess, Hahn & Ayala, 1956), the Bial and direct Ehrlich tests (Werner & Odin, 1952), sulphuric acid–acetic acid mixture (Hess, Coburn, Bates & Murphy, 1957) and resorcinol (Svennerholm, 1958). As these methods require heating with strong acids, they cannot distinguish free sialic acids from bound sialic acids, a differentiation necessary to follow the enzymic release of sialic acids from the sialomucoids. This paper describes two procedures, the thiobarbituric acid and the alkali–Ehrlich methods, which have been developed specifically for this purpose. A preliminary account of this work has already appeared (Aminoff, 1959).

A modification of the thiobarbituric acid method for the determination of sialic acid was developed independently by Warren (1959*a*, *b*). The work described here was carried out at about the same time, and there appear to be sufficient differences in the conditions of the reaction and the interpretation of the mechanism to warrant a detailed presentation.

## EXPERIMENTAL

### *Materials and methods*

*Derivatives of neuraminic acid.* *N*-Acetylneuraminic acid was isolated from bovine colostrum and crystallized from methanol. It had the following analytical values: decomposition temperature 171°, $[\alpha]_D^{26} - 36 \pm 1°$, Kjeldahl nitrogen 4·9% and equiv. wt. 299 (for $C_{11}H_{19}O_9N$ mol.wt. 309 and N 4·53%). Both *NO*-diacetylneuraminic acid isomers, obtained from bovine and equine submaxillary mucins (Blix, Lindberg, Odin & Werner, 1956), were kindly made available by Professor G. Blix. A sample of *N*-glycolylneuraminic acid, obtained from pig submaxillary mucin, was kindly provided by Professor E. Klenk. Methoxyneuraminic acid, prepared from bovine submaxillary mucin, was also a gift from Professor Klenk.

*Sialomucoids.* Through the courtesy of many investigators, samples of the following sialomucoids were made available: bovine salivary mucoid, ox serum mucoid, urine mucoid inhibitor (Dr R. Heimer), crystalline human serum $\alpha_1$-glycoprotein (Dr K. Schmidt) and colominic acid (Dr W. F. Goebel and Dr S. Roseman).

*Bovine colostrum sialomucoids.* Skimmed colostrum was exhaustively dialysed against distilled water, diluted with an equal volume of water and made 1 mM with respect to $CaCl_2$. The casein was removed by coagulation with commercial rennin and the supernatant treated with 2 vol. of acetone. The resulting precipitate was extracted with water and the fraction that was obtained between 33 and 50% (v/v) of ethanol contained most of the sialic acid. This material readily dissolved in water to give a clear solution and had 6–7% of sialic acid.

Collocalia *sialomucoids.* 'Edible bird's nest' is predominantly a salivary mucoid with which the *Collocalia* species of swift builds its nest. It is a commercially available oriental delicacy. Insoluble in water, it is readily solubilized by treatment of the finely ground nest with ficin (Lawton, McLoughlin & Morgan, 1956). The solubilized nest was heated in a boiling-water bath for 10 min. to denature extraneous proteins, and the supernatant fluid dialysed to remove low-molecular-weight components and then freeze-dried. The preparations were electrophoretically heterogeneous and contained 10–12% of sialic acid.

Application of Hestrin's (1949) hydroxylamine test for esters and lactones indicated the presence of such groups in colominic acid and Blix's bovine sialic acid, but none in *N*-acetylneuraminic acid or the sialomucoids from bovine colostrum or *Collocalia* salivary mucin.

### *Determination of* N*-acetylneuraminic acid by the thiobarbituric acid method*

*Reagents.* (*a*) Periodate: 25 mM-periodic acid in 0·125N-$H_2SO_4$ (pH 1·2). (*b*) Sodium arsenite: 2% solution of sodium arsenite in 0·5N-HCl. (*c*) Thiobarbituric acid: 0·1M solution of 2-thiobarbituric acid (Eastman Organic Chemicals Department, Eastman Kodak Co.) in water, adjusted to pH 9·0 with NaOH. It keeps well for about a month in a dark bottle at 4°. (*d*) Acid butanol: butan-1-ol containing 5% (v/v) of 12N-HCl.

* Present address: The Rackham Arthritis Research Unit, and The Department of Biological Chemistry, The University of Michigan, Ann Arbor, Mich., U.S.A.

*Procedure.* A solution of the sample, blank or standard (containing 5–40 $\mu$g. of *N*-acetylneuraminic acid) in 0·5 ml. of water is treated with 0·25 ml. of the periodate reagent for 30 min. in a water bath at 37°. The excess of periodate is then reduced with 0·2 ml. of the sodium arsenite. As soon as the yellow colour of the liberated iodine has disappeared (1–2 min.), 2 ml. of the thiobarbituric acid reagent is added and the test sample is covered and heated in a boiling-water bath for 7·5 min. The coloured solutions are then cooled in ice–water and shaken with 5 ml. of the acid butanol. The separation of the two phases is facilitated by a short, rapid centrifuging and the intensities of the colours in the butanol layer are compared at 549 m$\mu$ in a spectrophotometer. Despite the apparent discoloration on standing, the extinctions are constant for 2 hr. They decline appreciably in 20 hr.

The absorption spectrum of the colour obtained with *N*-acetylneuraminic acid is identical with that obtained by Warren (1959*b*). The molar extinction at 549 m$\mu$ is $70{\cdot}7 \times 10^3$, and the extinction is directly proportional to the concentration of *N*-acetylneuraminic acid.

The oxidation with periodate, resulting in the formation of the chromogen, is more rapid at 37° than at room temperature, and the final colour intensity is dependent on both the pH and the period of oxidation (Fig. 1). The excess of periodate is best removed with acid arsenite; alkaline arsenite, ethylene glycol, glycerol or glucose are not satisfactory as they interfere in the subsequent colour development. The intensity of the colour obtained on heating with thiobarbituric acid is independent of the pH of the reagent over the range 7–10. An alkaline reagent is preferred in order to increase the solubility of the thiobarbituric acid in water, a concentration of 0·1 M being necessary for the maximum colour production.

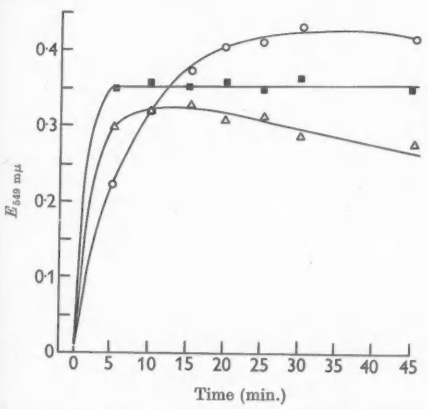



Fig. 1. Effect of the pH of the periodate and the period of oxidation at 37° on the amount of chromogen formed: ○, at pH 1·2; ■, at pH 7·1; △, at pH 8·5.

The coloured complex is both more stable and more soluble in acidified butan-1-ol. The absorption peak of the coloured material is sharper at 549 m$\mu$, and more intense in the butan-1-ol than in the aqueous phase before extraction.

*Specificity of the thiobarbituric acid method*

*Sialomucoids.* All the sialomucoids tested give little or no colour. Any interference in the test due to opalescence and discoloration is usually eliminated by the extraction with butan-1-ol.

*2-Oxo-3-deoxy sugar acids.* Weissbach & Hurwitz (1959) reported that the thiobarbituric acid assay is an excellent method for the estimation of 2-oxo-3-deoxy sugar acids. Under the conditions outlined above, these sugars gave an intense pink colour with the peak also at 549 m$\mu$. Thus a molar extinction of $72 \times 10^3$ was obtained with 2-oxo-3-deoxygluconic acid (a gift of Dr E. Racker).

*Deoxyribose, 2-aminopyrimidine and sulphadiazine.* Waravdekar & Saslaw (1957, 1959) were the first to apply the known reactivity of thiobarbituric acid with aldehydes (Patton & Kurtz, 1951) to the determination of 2-deoxyribose after oxidation with periodate. The periodate oxidation products of both 2-aminopyrimidine and sulphadiazine have also been shown to react (Kohn, 1945; Shepherd, 1948). Under the present conditions, these compounds react to give intense colours with the maximum light-absorption also at 532 m$\mu$. The molar extinction of the colour given by deoxyribose was $124 \times 10^3$.

All of these compounds will therefore interfere in the estimation of *N*-acetylneuraminic acid.

*Miscellaneous compounds.* The following substances were tested and gave no colour: *N*-acetylglucosamine (1 mg.), *N*-acetylmuramic acid (50 $\mu$g.), arabinose (1 mg.), ascorbic acid (0·1 mg.), aspartic acid (0·1 mg.), fructose (0·2 mg.), $\gamma$-galactonic lactone (0·2 mg.), $\delta$-gluconolactone (0·2 mg.), glucosamine–HCl (1 mg.), glucosaminic acid (1 mg.), histidine–HCl (1 mg.), pyrrole-2-carboxylic acid (100 $\mu$g.), ribose 5-phosphate (0·1 mg.), serine (0·1 mg.), streptomycin sulphate (1 mg.), sucrose (0·1 mg.), tartaric acid (0·2 mg.) and threonine (1 mg.).

Under the conditions outlined above, *N*-acetylglucosamine gives no colour. However, when tested in the procedure of Waravdekar & Saslaw (1957) and of Weissbach & Hurwitz (1959), a faint colour was obtained with the maximum absorption of light at 550 m$\mu$. Glucosamine–HCl under the same conditions gave no colour.

*Nature of the chromogen*

Neither formaldehyde nor formic acid gives a pink colour with thiobarbituric acid under the conditions of the test. The observations of Wilbur, Bernheim & Shapiro (1949) would implicate glyoxylic acid as a possible chromogen. However, glyoxylic acid, obtained by the reduction of oxalic acid with magnesium turnings (Benedict, 1909) or by the oxidation of tartaric acid with periodate (Fleury & Bon-Bernatets, 1936), gave no colour under the conditions of the test.

The coloured complexes of the oxidation products of *N*-acetylneuraminic acid and 2-oxo-3-deoxygluconic acid migrated at the same rate on paper chromatography (with acidified 2-ethoxyethylacetate, *cyclo*hexanone or butan-1-ol as solvent) and on paper electrophoresis at pH 3·45 (citrate–HCl buffer), pH 8·4 (borate buffer) and pH 8·6 (veronal buffer), where the migration was anodic. In contrast, the coloured complex obtained with 2-deoxyribose is neutral and immobile on paper electrophoresis, and on paper chromatography it migrated faster than did the coloured complexes of *N*-acetylneuraminic acid and 2-oxo-3-deoxy sugar acids.

These results imply an identity of the coloured complexes obtained, and hence also of the chromogenic oxidation products obtained with *N*-acetylneuraminic acid and 2-oxo-3-deoxygluconic acid. Unfortunately, this could not be confirmed owing to the apparent lability of the chromogens on paper when tested by chromatography and electrophoresis.

Only 6–16 % of the total nitrogen was released as $NH_3$ as a direct consequence of the oxidation of *N*-acetylneuraminic acid for 20 min. at 37° with 25 mM-periodate in 0·125 N-$H_2SO_4$.

*Determination of* N*-acetylneuraminic acid by the alkali–Ehrlich method*

This reaction, obtained with the sialic acids but not with sialomucoids, will be referred to as the alkali–Ehrlich reaction to avoid confusion, and to distinguish it from the many colorimetric reactions involving the use of Ehrlich reagent, such as the Elson & Morgan (1933) method for hexosamines, Morgan & Elson (1934) test for *N*-acetylhexosamine, and the direct Ehrlich test (Werner & Odin, 1952), which does not distinguish free from bound sialic acid.

*Reagents.* (*a*) Borate buffer, pH 8·5: 0·2 M-boric acid solution is adjusted to pH 8·5 with NaOH. (*b*) Ehrlich reagent: 1·33 % solution of *p*-dimethylaminobenzaldehyde (Eastman Kodak Co.) is prepared in 95 % ethanol–analytical-grade conc. HCl (1:1, v/v) solvent. (*c*) 95 % Ethanol.

*Procedure.* To a solution of the sample, blank or standards (containing 10–100 $\mu$g. of *N*-acetylneuraminic acid) in 0·5 ml. of water, is added 0·5 ml. of 0·2 M-borate at pH 8·5. The solutions are heated at 100° for 45 min., cooled, treated with 3 ml. of ethanol followed by 1 ml. of the Ehrlich reagent and heated at 70° for a further 20 min. (in covered tubes) to develop the colour. Immediately after cooling, the extinctions are determined at 558 m$\mu$.

The absorption spectrum of the violet colour obtained with 100 $\mu$g. of *N*-acetylneuraminic acid is shown in Fig. 2. The molar extinction at 558 m$\mu$ is $7{\cdot}7 \times 10^3$, and the extinction is directly proportional to the concentration of *N*-acetylneuraminic acid.

The colour obtained is not stable, fading by some 10 % at the end of 1 hr. and with the absorption peak shifting to 560 m$\mu$.

Increasing concentrations of alkali result in a more rapid chromogen formation with, however, a concomitant loss in selectivity as sialomucoids also react. Buffered alkaline solutions are more suitable, and, of the buffers tested, borate gives the most intense colours. At pH 8·5 *N*-acetylneuraminic acid gives the maximum colour with the minimum interference from sialomucoids. At this pH, some 40–50 min. heating at 100° is necessary to obtain complete conversion of *N*-acetylneuraminic acid into the chromogen.

A variation in the concentration of *p*-dimethylaminobenzaldehyde from 1·0 to 2·5 % in the

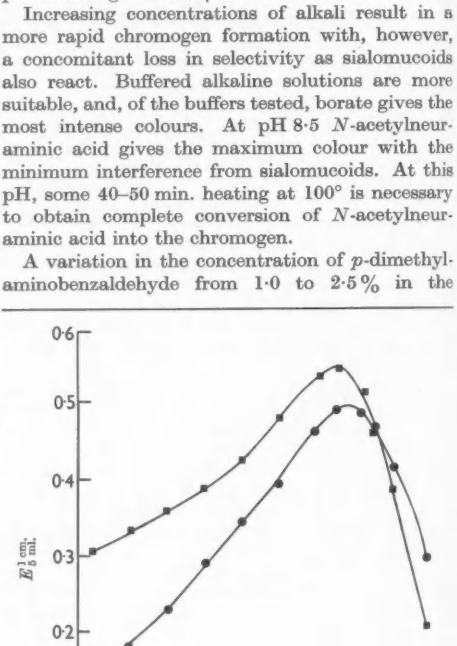



Fig. 2. Absorption spectra of the coloured complexes obtained with (●) *N*-acetylneuraminic acid (100 $\mu$g.) and (■) 2-carboxypyrrole (1 $\mu$g.) in the alkali–Ehrlich procedure.

Ehrlich reagent has no effect on the intensity of the colour obtained. The concentration of 1·33% was used for convenience, as the same reagent is used in the estimation of hexosamines (Elson & Morgan, 1933). Less acid in the Ehrlich reagent results in slower colour development; stronger acid gives rise to a more-labile coloured complex.

*Specificity of the alkali–Ehrlich method*

*Sialomucoids.* Most of the sialomucoids tested give a negligible amount of colour.

*2-Carboxypyrrole.* An immediate and intense purple colour is obtained on adding the Ehrlich reagent to 2-carboxypyrrole in the cold. In the procedure for estimation of *N*-acetylneuraminic acid, 2-carboxypyrrole gives a pinker colour and the two absorption spectra are quite distinct, although both show peaks of absorption at 554–560 mμ (Fig. 2). The intensity of colour given by 2-carboxypyrrole at 555 mμ is equivalent to a molar extinction of $309 \times 10^3$.

*Hexosamines and derivatives.* Glucosamine–HCl (1 mg.), glucosaminic acid (1 mg.) and *N*-acetylmuramic acid (50 μg.) give no colour at all. *N*-Acetylglucosamine gives but a trace of colour, equivalent to 0·014 mole of *N*-acetylneuraminic acid.

*Miscellaneous.* The following substances in 1 mg. amounts give no detectable colour: L-glutamine, D-glutamic acid, L-histidine, 2-oxo-3-deoxygluconic acid (23 μg.), proline and tryptophan. L-Hydroxyproline gives a very faint pink colour with a peak of absorption at 560 mμ, and 1 mole gives an intensity equivalent to 0·01 mole of *N*-acetylneuraminic acid. Indole gives a strong pink colour with cold Ehrlich reagent, which intensifies on heating at 70°. Under the conditions of the test, it gives a double-peaked absorption curve with maxima at 540 and 560 mμ, and an intensity equivalent to a molar extinction of 146 and $159 \times 10^3$ respectively.

Neither fructose (1 mg.) nor lysine–HCl (0·1 mg.) alone or in admixture in this proportion gives any colour in this test, in contrast with the faint but distinct colours that they give in the hexosamine and *N*-acetylhexosamine tests (Immers & Vasseur, 1950, 1952; Aminoff, Morgan & Watkins, 1952).

*Nature of the chromogen*

Heating with the borate buffer at pH 8·5 converts the *N*-acetylneuraminic acid into a derivative with marked absorption in the ultraviolet. Even though the u.v. absorption of the heated product continues to rise fairly uniformly throughout the 105 min. investigated, the rate of colour formation resulting from the addition of the Ehrlich reagent indicates a diphasic reaction with the point of inflexion after approx. 25 min. heating with the borate (Fig. 3). The u.v.-absorption spectra of the solutions of *N*-acetylneuraminic acid for various periods of heating are shown in Fig. 4. They appear similar to that given by 2-carboxypyrrole in 0·1 M-borate, pH 8·5, with the maximum absorption at 257 mμ and a molecular extinction of 11 500.

The chromogens obtained on heating *N*-acetylneuraminic acid with borate buffer were examined by paper electrophoresis and partition chromatography. Ehrlich reagent (as modified by Partridge, 1948) was used to spray the strips and, as has been previously observed by Berggård & Odin (1958), butanol–acetic acid–water (4:1:5) gives the best resolution. With this solvent system, at least four visible spots were obtained with $R_F$ 0·14, 0·41, 0·67 and 0·87. The chromogen with $R_F$ 0·41 predominates in the material heated for 45 min. and decreases with the longer heating periods. It would thus appear to be the principal chromogen involved in the alkali–Ehrlich estimation.

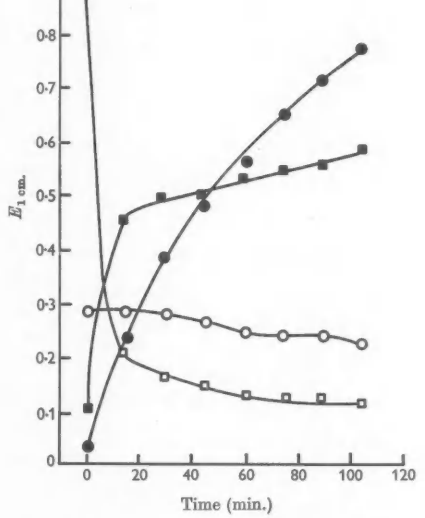



Fig. 3. Effect of the period of heating of *N*-acetylneuraminic acid in borate buffer, pH 8·5 (0·1 M), on: ●, *E* at 260 mμ; ■, subsequent colour development (at 558 mμ) on the addition of the Ehrlich reagent as in the alkali–Ehrlich procedure. ○, □, Stability of the sialic acid under these conditions as determined by the resorcinol and thiobarbituric acid procedures respectively.

Fig. 4. Absorption spectra of *N*-acetylneuraminic acid (100 μg.) heated at 100° for various periods in 0·1 M-borate buffer, pH 8·5: ▲, 15 min.; ○, 42 min.; ■, 105 min.

Table 1. *Comparison of the reactivity of various derivatives of neuraminic acid in the thiobarbituric acid and alkali–Ehrlich determinations*

Conditions of the determinations are described in the text. Derivatives of neuraminic acid were used without prior drying over $P_2O_5$.

| Substance | $10^{-3}\,\epsilon$: Thiobarbituric method (549 mμ) | $10^{-3}\,\epsilon$: Alkali–Ehrlich method (558 mμ) |
|---|---|---|
| *N*-Acetylneuraminic acid | 70·7 | 7·7 |
| *N*-Glycolylneuraminic acid | 44·4 | 7·9 |
| *NO*-Diacetylneuraminic acids: | | |
| (*a*) Bovine | 0 | 6·1 |
| (*b*) Equine | 65·4 | 6·1 |
| Methoxyneuraminic acid | 0 | 0 |

*Applications of the two methods of determining N-acetylneuraminic acid*

*Comparison of derivatives of neuraminic acid.* The results obtained with the various derivatives of neuraminic acid are expressed in terms of molecular extinction and summarized in Table 1. The reactivity of bovine sialic acid in the thiobarbituric acid test can be greatly increased on removal of the *O*-acetyl groups by cold alkali or mild acid hydrolysis (1 hr. with 0·01 N-HCl at 84°). The reactivity of the bovine sialic acid in the alkali–Ehrlich procedure can be attributed to the rapid hydrolysis of the *O*-acetyl group under the alkaline conditions of the test.

*Stability of N-acetylneuraminic acid to acid and alkali.* Table 2 summarizes the results obtained on treatment of *N*-acetylneuraminic acid with acid. Confirming the observations of Svennerholm (1958), *N*-acetylneuraminic acid appears to be fairly stable when heated in 0·1 N-HCl at 84° as determined by the resorcinol method. However, when assayed by the thiobarbituric acid and alkali–Ehrlich methods, there are distinct indications of degradation, the moiety essential for a positive colour with thiobarbituric acid being the more labile. With 0·01 N-HCl at 100° the degradation is more enhanced. Neither the original solution of *N*-acetylneuraminic acid nor the resulting acid-treated material reacts with hydroxylamine, and the possibility of lactone formation on heating with the HCl is thereby eliminated.

The thiobarbituric acid-reacting moiety of *N*-acetylneuraminic acid is also extremely unstable in alkali, as seen in Fig. 3. Again this marked lability of *N*-acetylneuraminic acid to alkali is not as readily detected by the resorcinol method.

*Rate of release of N-acetylneuraminic acid from sialomucoids on acid hydrolysis.* Three sialomucoids were chosen for this study: solubilized *Collocalia* mucoid, the sialomucoids from bovine colostrum and colominic acid, presumably a polymer of sialic acid (Barry, 1958).

Table 2. *Stability of N-acetylneuraminic acid to acid as determined by the resorcinol, alkali–Ehrlich and thiobarbituric acid methods*

In each case the colour produced is expressed as a percentage of that given by the untreated substance.

| Period of heating (min.) | 0·1 N-HCl at 84°: Resorcinol (580 mμ) | 0·1 N-HCl at 84°: Alkali–Ehrlich (558 mμ) | 0·1 N-HCl at 84°: Thiobarbituric acid (549 mμ) | 0·01 N-HCl at 100°: Resorcinol (580 mμ) | 0·01 N-HCl at 100°: Alkali–Ehrlich (558 mμ) | 0·01 N-HCl at 100°: Thiobarbituric acid (549 mμ) |
|---|---|---|---|---|---|---|
| 5 | 99 | 99 | 100 | 105 | 99 | 100 |
| 30 | 95 | 92 | 98 | 98 | 100 | 80 |
| 60 | 96 | 80 | 83 | 98 | 91 | 65 |
| 120 | 88 | 81 | 77 | 82 | 84 | 44 |
| 240 | 80 | 71 | 61 | 58 | 65 | 26 |

The rate of release of sialic acid from the sialomucoid of *Collocalia* by acid hydrolysis is almost the same when determined by the thiobarbituric acid or alkali–Ehrlich procedure (Fig. 5). A similar behaviour was also observed with bovine colostrum. However, with colominic acid, there is an apparently greater release of *N*-acetylneuraminic acid as measured by the alkali–Ehrlich method (Fig. 5). This suggests that the sialic acid, as released from colominic acid by mild acid hydrolysis, is still substituted in such a way that it does not give the full colour with the thiobarbituric acid assay. This substituent is presumably alkali-labile and thereby does not interfere in the alkali–Ehrlich determination. The positive hydroxylamine test given by colominic acid and the isolation of *NO*-diacetylneuraminic acid (the bovine isomer) from the hydrolysis products of the endotoxins of *Escherichia coli* (DeWitt & Rowe, 1959) suggest that this substituent is the *O*-acetyl group.

Two further points emerge from these studies and are illustrated in Fig. 6. First, the sialic acid of *Collocalia* mucoid, in contrast with that of colostrum or colominic acid, appears to be rapidly degraded by the acid when assayed by the thiobarbituric acid method. Secondly, this rapid degradation is not reflected in the resorcinol and alkali–Ehrlich procedures (see also Table 2), which would suggest that the thiobarbituric acid assay involves a more 'labile' moiety of the sialic acid molecule.

*Release of sialic acid from sialomucoids by the sialidase of* Vibrio cholera. A partially purified specimen of sialidase prepared from *Vibrio cholera* was used for these studies. The rate of release of sialic acid from the sialomucoids of *Collocalia* salivary mucin and bovine colostrum is shown in Fig. 7. The course of hydrolysis was followed by the thiobarbituric acid and the alkali–Ehrlich procedures. There is a rapid release of sialic acid, with little or no subsequent loss of free sialic acid, indicating the absence of a *N*-acetylneuraminic acid aldolase (Comb & Roseman, 1958) in this preparation of the sialidase.

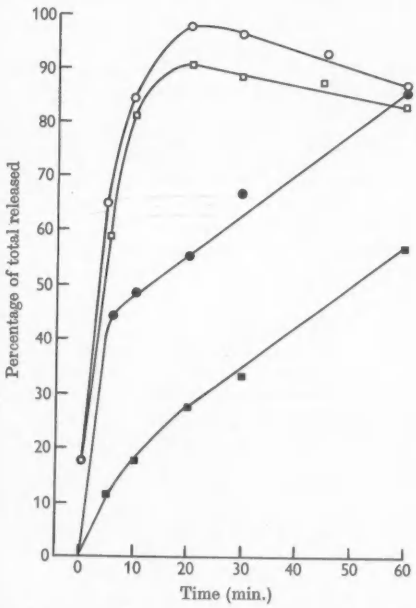



Fig. 5. Rate of release of *N*-acetylneuraminic acid by hydrolysis with 0·1 N-HCl at 84° from *Collocalia* mucoid as determined by alkali–Ehrlich (○) and thiobarbituric, acid (□) procedures; and from colominic acid by alkali–Ehrlich (●) and thiobarbituric acid (■) procedures.

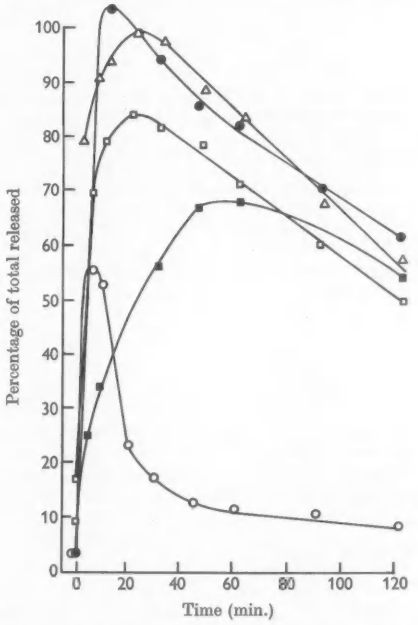



Fig. 6. Rate of release of *N*-acetylneuraminic acid by hydrolysis with 0·01 N-HCl at 100° as determined by the thiobarbituric acid procedure. ●, Bovine colostrum; ■, colominic acid; ○, *Collocalia* sialomucoids; △, □, sialic acid content of the *Collocalia* mucoid simultaneously determined by the resorcinol and alkali–Ehrlich procedures.

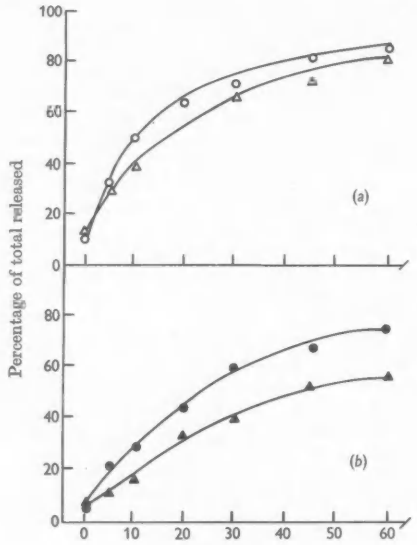



Fig. 7. Rate of release of *N*-acetylneuraminic acid by hydrolysis at 37° with the *Vibrio cholera* sialidase. (*a*) *Collocalia*, (*b*) bovine colostrum sialomucoids, as determined by (○, ●) the thiobarbituric acid and (△, ▲) the alkali–Ehrlich procedures.

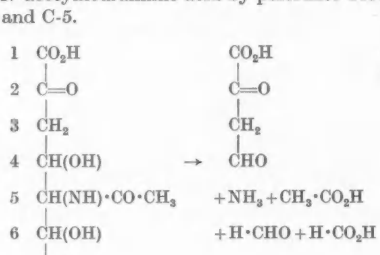
Both methoxyneuraminic acid and colominic acid were unaffected by the sialidase of *Vibrio cholera*, as determined by either the thiobarbituric acid or alkali–Ehrlich procedure.

## DISCUSSION

Two alternative methods have been described for the determination of free sialic acid. Together with the classical methods for the assay of total sialic acid (direct Ehrlich test, Bial, resorcinol etc.), it is now possible to determine the total, bound and free, sialic acid in a mixture, and to follow the hydrolysis and degradation of sialic acid from sialomucoids.

The thiobarbituric acid method as outlined here is slightly more sensitive than is the method described by Warren (1959*b*), and, moreover, experience with the two methods has indicated a greater freedom from interference in complex biological systems (S. Roseman, personal communication). A study of the conditions of assay has indicated that the high acidity of the periodate reagent as recommended by Warren (1959*b*) is not essential to obtain a colour. Indeed, a more selective choice of conditions of oxidation can be more informative. For instance (*a*) in the assay here presented, the thiobarbituric acid method can distinguish *N*-acetylneuraminic acid from the bovine *NO*-diacetylneuraminic acid, and (*b*) oxidation at pH 1·2 and 7 will distinguish between acid-labile compounds and free sialic acid. Thus the cytidine monophosphate derivative of sialic acid (Comb, Shimizu & Roseman, 1959) reacts quantitatively when oxidized at pH 1·2, and gives but a trace of colour when oxidized at pH 7, whereas sialic acid still gives an intense colour (Fig. 1).

The evidence presented above indicates that the coloured complex given by *N*-acetylneuraminic acid in the thiobarbituric acid procedure is identical with that obtained with 2-oxo-3-deoxy sugar acids. According to Weissbach & Hurwitz (1959) and Srinivasan & Sprinson (1959), β-formyl pyruvate is the periodate oxidation product of 2-oxo-3-deoxy sugar acids responsible for giving a colour with the thiobarbituric acid reagent. One should therefore postulate an oxidative cleavage of the *N*-acetylneuraminic acid by periodate between C-4 and C-5.




It is difficult, however, to reconcile this interpretation of the mechanism of the reaction with the behaviour of certain derivatives of neuraminic acid in the thiobarbituric acid procedure. The studies of Blix *et al.* (1956) and Blix (1958) on the structure of the sialic acids suggest that the *O*-acetyl group in the bovine sialic acid is located at C-7, and in the equine isomer at C-4. Contrary to expectation, the equine isomer gave substantial colour whereas the bovine isomer gave no colour at all. Preliminary *O*-deacetylation of the bovine sialic acid by treatment with alkali at room temperature, or by mild acid hydrolysis, results in complete reactivity in the thiobarbituric acid procedure.

The absorption spectrum of the colour obtained by Gottschalk & Lind (1949), under conditions somewhat similar to those used here in the alkali-Ehrlich test, would seem to suggest that the

chromogen obtained might be related to that given by *N*-acetylglucosamine in the Morgan & Elson (1934) test. The subsequent identification of *N*-acetylhexosamine in the alkaline-degradation products of *N*-acetylneuraminic acid by Kuhn & Brossmer (1956) and Zilliken & Glick (1956) would appear to substantiate this possibility. However, the evidence presented here indicates that we are dealing with a different chromogen. The absorption spectrum in both the ultraviolet and visible range for *N*-acetylneuraminic acid and *N*-acetylglucosamine (Aminoff *et al.* 1952) are quite distinct. Moreover, *N*-acetylglucosamine hardly reacts in the alkali–Ehrlich test and, conversely, *N*-acetylneuraminic acid gives no colour in the test for *N*-acetylhexosamines.

Although the evidence reported here, and in the literature, strongly suggests that pyrrole-2-carboxylic acid is one of the end products of the alkaline degradation of sialic acid, the nature of the immediate chromogens obtained in the alkali–Ehrlich method of determining *N*-acetylneuraminic acid is still unresolved. More than one chromogen is formed, and the principal chromogen obtained after 45 min. heating in the borate buffer has $R_F$ 0·41 in butan-1-ol–acetic acid–water (4:1:5).

The thiobarbituric acid and the alkali–Ehrlich procedures for the determination of sialic acid are based on different principles, and appear to involve different moieties of the sialic acid molecule. Of the two, the thiobarbituric acid method is superior, not only for its greater sensitivity, but also qualitatively for its greater facility in detecting possible modifications of the sialic acid molecule.

However, following the kinetics of mild acid hydrolysis of sialomucoids by both methods can, as has been illustrated, reveal certain aspects of the structure of the complex mucoids. The results obtained, moreover, emphasize the danger of using the thiobarbituric acid procedure alone to determine the sialic acid content of mucoids, as has been suggested by Warren (1959*b*).

## SUMMARY

1. Two colorimetric procedures, the thiobarbituric acid and alkali–Ehrlich, have been developed for the determination of free sialic acid in the presence of bound sialic acid.

2. These methods apparently involve reactions with different moieties of the molecule.

3. Complementing each other and the existing methods of determining total sialic acid in sialomucoids, they serve as valuable tools in the study of the degradation and release of sialic acids from sialomucoids.

4. The structural implications derived from the application of these tests are briefly discussed.

The author wishes to express his gratitude to the many individuals who have donated samples of sialomucoids and derivatives of neuraminic acid, to colleagues at work and, more especially, to Dr Saul Roseman for interesting and helpful discussions, and finally to Mr Julius Schachter for his technical assistance.

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# Studies on Carbohydrate-Metabolizing Enzymes

## 7. YEAST ISOAMYLASE*

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Although the starch- and glycogen-metabolizing enzymes of higher plants and animals have been intensively studied [for reviews, see Manners (1953) and Whelan (1958)], the related yeast enzymes have attracted comparatively little attention. For example, the presence of phosphorylase in yeast extracts was reported by Schaffner & Specht (1938) and Cori, Colowick & Cori (1938), but the enzyme does not appear to have been highly purified, whereas both potato and muscle phosphorylase have been crystallized. Further, the properties of yeast branching enzyme have only recently been reported (Gunja, Manners & Khin Maung, 1960), although potato Q-enzyme was first investigated 16 years ago.

This paper is concerned with a yeast 'debranching' enzyme, hereafter referred to as isoamylase, which hydrolyses the outer $\alpha$-(1 $\rightarrow$ 6)-glucosidic inter-chain linkages in amylopectin and glycogen. Nishimura (1931) noted that yeast extracts contained an enzyme, 'amylosynthease', which could liquefy starch and accelerate the action of normal amylases, although it differed in thermolability from $\alpha$- and $\beta$-amylases. Further studies by Nishimura & Minagawa (1931) and Minagawa (1932) showed that enzyme action on glutinous rice starch, which was optimum at pH 6·2 and 20°, caused an increase in iodine-staining power. Amylosynthease was therefore regarded as a starch-synthesizing enzyme. Later, Meyer & Bernfeld (1942) observed that yeast extracts attacked both starch and its $\beta$-dextrin. A small increase (7–15 %) in the $\beta$-amylolysis limit of the latter was noted. The impure enzyme preparation also hydrolysed maltose and isomaltose. The action of amylosynthease on rice starch was re-examined by Maruo & Kobayashi (1951). The product had a lower molecular weight, higher $\beta$-amylolysis limit, stained bluish-purple with iodine, and showed a tendency to retrograde from solution. These results were considered to be caused by extensive debranching of the starch, and the new name isoamylase was proposed for the enzyme. This implies a starch-degrading function. This activity is similar to that of R-enzyme on amylopectin (Hobson, Whelan & Peat, 1951).

In view of our interest in the structure and metabolism of glycogen (Manners, 1957), the action of yeast isoamylase on this and related polysaccharides was investigated. It should be noted that R-enzyme has no action on normal glycogen (Peat, Whelan, Hobson & Thomas, 1954; Fleming & Manners, 1958). As stated in the preliminary account of this work (Manners & Khin Maung, 1955) we first encountered isoamylase as an impurity in yeast-phosphorylase preparations.

* Part 6: Liddle, Manners & Wright (1961).

## METHODS AND MATERIALS

*Analytical methods.* The methods used for paper chromatography, the determination of reducing sugars and the analysis of polysaccharides (by $\alpha$- and $\beta$-amylolysis and periodate oxidation) have been described (Gunja *et al.* 1960).

*Determination of isoamylase activity.* The action of a debranching enzyme causes an increase in both the $\beta$-amylolysis limit, and iodine-staining power of glycogen but no appreciable increase in reducing power. The changes may be used as a measure of activity although since neither property is directly related to the number of $\alpha$-(1$\rightarrow$6)-glucosidic linkages hydrolysed, such values are relative rather than absolute.

Digests containing approximately equal weights of glycogen and isoamylase preparation (usually 1 or 2 mg./ml. in a total volume of 25 ml.) were incubated at pH 5·9

and room temperature (18–20°) for 18 hr. Unless otherwise stated, 0·2M-sodium acetate buffer, pH 5·9 or 6·0, was used. For β-amylolysis, a portion was heated (3 min. at 98°) and then cooled, and β-amylase added; the maltose content was then measured after incubation for 24 hr. at 35°. For iodine-staining, samples (2 or 3 ml.) were stained with iodine solution [0·2% of iodine in 2% (w/v) potassium iodide; 1 or 2 ml.], acidified (3 drops of 5N-hydrochloric acid) and diluted to 25 ml. with water, and the extinction was measured on a Unicam SP. 500 spectrophotometer at a wavelength in the region 425–480 mμ (depending upon the wavelength of maximum absorption of the particular glycogen sample) against an iodine–water blank.

*Enzyme preparations.* The samples of pressed brewer's yeast (*Saccharomyces cerevisiae*) and α- and β-amylase were those used by Gunja *et al.* (1960).

*Substrates.* Glycogen and amylopectin samples were as described by Gunja *et al.* (1960). Maltodextrin (a mixture of linear and branched maltosaccharides produced by the action of malt α-amylase on potato starch) was kindly provided by Professor C. S. Hanes, F.R.S.

*Nomenclature.* In multiply branched molecules of the glycogen–amylopectin type, A-chains are defined as those which are attached to the rest of the molecule by a single linkage from the reducing group, whereas B-chains, which are similarly linked, also have other chains attached to them (Peat, Whelan & Thomas, 1952). (The single reducing group in the molecule is carried by a C-chain; this can be neglected in the present study.)

### *Preparation of isoamylase*

*Acetone fractionation.* Dried yeast (200 g.) was extracted at room temperature with 0·1M-sodium bicarbonate solution (1000 ml.) for 2 hr. All subsequent operations were carried out at low temperatures (−4° or −7°). After centrifuging (1500*g*; 20 min.; −4°), the supernatant solution (580 ml.) was treated with an equal volume of cold A.R. acetone at −7°. The precipitate was collected (centrifuging at −4°), and triturated for several hours with 0·2M-sodium citrate buffer (pH 5·8; 500 ml.). Insoluble material was discarded, and the solution (480 ml.) fractionated with acetone at −7°. The protein fractions precipitated in acetone concentrations 0–20, 20–33 and 33–42% (v/v) were collected, washed with cold acetone, and dried *in vacuo* at 0° over phosphorus pentoxide, giving respectively fractions 1–3. The residue from the first extraction was re-extracted with bicarbonate solution (500 ml.), and to the solution cold acetone to 50% (v/v) concentration was added, giving fraction 4.

The isoamylase activities of the four fractions, expressed as the percentage increase in the β-amylolysis limit of glycogen were 8, 48, 10 and 6 respectively. Fraction 3 contained the greatest amount of yeast branching enzyme and maltase.

Fraction 2 could be used for isoamylolysis without further purification in certain experiments, e.g. the action on glycogen as followed by β-amylolysis or iodine staining. However, in other experiments, the contaminating yeast branching enzyme and maltase interfered, and had, therefore, to be eliminated.

*Further purification of isoamylase.* (*a*) Elimination of yeast branching enzyme. A starch-adsorption method similar to that developed by Hobson *et al.* (1951) for the removal of α-amylase and Q-enzyme from potato and bean preparations was used. Isoamylase (fraction 2; 5 g.) was dissolved in aqueous 20% ethanol solution (200 ml.) at −4°; anhydrous sodium sulphate (2 g.) was added and the solution stirred slowly with cooling to −7°. Maize starch (112 g.) was added to the solution, and stirring continued for 2 hr. After centrifuging at −4°, cold acetone (180 ml.) was added to the supernatant solution (90 ml.). The precipitate was collected at −4°, washed twice with cold acetone and dried under vacuum. The yield was 2·1 g.

This procedure gave an isoamylase preparation having little or no action on amylose. On incubation with potato amylose, the extinctions (680 mμ) of the iodine complex after 5 min., 2 and 24 hr., were 0·50, 0·50 and 0·41 respectively. The β-amylolysis limit of wrinkled-pea amylose was 75 and 77% respectively before and after treatment with isoamylase (Cowie, Fleming, Greenwood & Manners, 1957).

(*b*) Elimination of maltase. (i) *Acid treatment.* Fraction 2 (450 mg.) was dissolved in 0·2M-sodium acetate buffer (pH 4·5; 45 ml.) at room temperature and samples (5 ml.) were removed at intervals. The relative stabilities of isoamylase and maltase are given in Table 1.

Despite some loss in activity, the isoamylase could be purified by dissolving fraction 2 (1 g.) in buffer, pH 4·5 (100 ml.), for 10 hr. at 18°; after adjusting the pH to 5·9 and centrifuging, the protein (fraction 2*a*) was isolated by precipitation with cold acetone. The yield was 0·55 g.

On incubation of fraction 2*a* with maltose, at 20° and pH 5·9 for 23 hr., no increase in reducing power was observed [with either Somogyi (1952) or Phillips & Caldwell (1951) reagents] and glucose was not detected by paper chromatography. Under similar conditions, the β-amylolysis limit of glycogen and amylopectin β-dextrin increased by 25 and 23% respectively.

(ii) *Further acetone fractionation.* A bicarbonate extract of yeast (100 g.) was treated with acetone as before, except that all the material precipitated by 30% acetone concentration was collected (i.e. approx. fractions 1 and 2). This precipitate was redissolved in 0·2M-sodium citrate buffer (pH 6·0; 100 ml.), insoluble material removed by centrifuging, and the solution again fractionated with acetone. The protein (fraction 5) obtained between 33 and 50% acetone concentration was collected, washed and dried. Yield, 8·0 g.

Fraction 5 had no action on maltose or maltotriose, but slowly hydrolysed isomaltose. On incubation (0·2% solution) with glycogen (0·1% solution) at pH 6·0 and 20°, the extinction of the polysaccharide–iodine complex at 440 mμ increased by 13, 28 and 39% after 2, 4 and 24 hr. respectively.

Table 1. *Stability of isoamylase and maltase at* pH 4·5 *and* 18°

Maltase activity is expressed as apparent hydrolysis of maltose (%). Isoamylase activity is expressed as increase in the β-amylolysis limit of glycogen (%).

| Time of incubation (hr.) | Maltase activity | Isoamylase activity |
|---|---|---|
| 4 | 40 | 50 |
| 6 | 31 | — |
| 7 | 22 | 48 |
| 8 | 6 | 41 |
| 10 | 0 | 36 |

## RESULTS

### *Properties of isoamylase*

*Effect of enzyme concentration.* When digests of glycogen and various amounts of isoamylase were examined by iodine-staining after 2 and 6 hr., a linear relationship between enzyme concentration and the percentage increase in extinction of the polysaccharide–iodine complex was observed.

A linear relationship was also obtained between enzyme concentration and the degree of hydrolysis of glycogen when various amounts of isoamylase were incubated with glycogen and a constant excess of $\beta$-amylase. Under these conditions, the extent of $\beta$-amylolysis is limited by the concentration of isoamylase.

*Effect of temperature and* pH. Isoamylase is relatively thermolabile. After incubation at various temperatures for 1 hr., the activity (expressed as the increase in $\beta$-amylolysis limit of glycogen) was 15 at 35°, 11 at 40°, 5 at 45°, 4 at 50° and 2 at 55°. These results may be contrasted with those obtained on incubation of fraction 2 with maltose; the relative activity (expressed as the apparent increase in reducing power) was 7·3, 7·1 and 0·1 at 35°, 45° and 55° respectively.

By iodine-staining measurements, the optimum temperature was about 25°, the extinction of a glycogen–iodine complex increased by 0·134, 0·143, 0·142 and 0·122, after incubation at 21, 25, 29 and 37° for 1·5 hr.; after 7 hr., the corresponding figures were 0·142, 0·153, 0·151 and 0·143.

In sodium acetate buffer, activity was optimum at about pH 6·0, as determined by both iodine-staining and $\beta$-amylolysis-limit methods.

In B.D.H. Universal buffer (British Drug Houses Ltd.) (see later) isoamylase was inactive.

*Effect of various inhibitors and activators.* In the following experiments, the action on glycogen was followed by iodine-staining, and the results are expressed as the percentage increase in extinction at 460 m$\mu$.

(*a*) Ammonium molybdate. This substance, which inhibits R-enzyme (Whelan, 1958), also caused a marked reduction in isoamylase activity. In the presence of 0, 0·2 and 2·0 % (w/v) of ammonium molybdate, the activities after 4 hr. were 78, 19 and 4 respectively, and, after 24 hr., 95, 41 and 8.

(*b*) Other inorganic salts. The effect of various salts which alter the activity of other starch-metabolizing enzymes (e.g. $\alpha$- and $\beta$-amylase, Q-enzyme) was examined. The results are shown in Table 2, and suggest that sodium borate is the inhibiting component of B.D.H. Universal buffer.

(*c*) Gluconolactone. This lactone has a pronounced effect on many carbohydrases (Conchie & Levvy, 1957; Reese & Mandels, 1959) and, as shown in Table 2, completely inhibits isoamylase at a concentration of 10 mM.

(*d*) Sulphydryl reactants. Isoamylase action was only partly inhibited by SH-group reactants as shown in Table 3.

### *Specificity of isoamylase*

The partially purified isoamylase preparation (fraction 5) had no hydrolytic activity on the $\alpha$-(1 → 4)-glucosidic linkages in maltose (by reducing-power measurements and paper chromatography), in maltotriose (by paper chromatography), or in amylose (by iodine-staining and $\beta$-amylolysis). On incubation with glycogen the increase in reducing power was extremely small; in one experiment this corresponded to about 7 % apparent conversion into maltose.

*Action on $\alpha$-(1 → 3)-glucosidic linkages.* Isolichenin, a linear polysaccharide containing 40 % of $\alpha$-(1 → 4)- and 60 % of $\alpha$-(1 → 3)-glucosidic linkages (Chanda, Hirst & Manners, 1957), was treated with salivary $\alpha$-amylase as follows: isolichenin (47·5 mg.), $\alpha$-amylase (5 mg.; 200 units), 0·1 M-sodium chloride (5 ml.) and water (to 25 ml.) were incubated at 35° for 24 hr. The increase in reducing power corresponded to 9·7 % apparent conversion into

Table 2. *Effect of salts and gluconolactone on isoamylase activity*

Digests containing glycogen (25 mg.), isoamylase (50 mg.), 0·2 M-sodium acetate buffer (pH 6·0; 5 ml.) and inhibitor (1 ml.) in a total volume of 10 ml. were incubated at 20° for 15 hr.

| Inhibitor | Final concn. (mM) | Activity (increase in extinction, %) |
|---|---|---|
| — | — | 54 |
| Mercuric chloride | 0·1 | 1 |
| Magnesium chloride | 20 | 20 |
| Sodium borate | 10 | 1 |
| Sodium chloride | 20 | 17 |
| Sodium fluoride | 10 | 33 |
| Gluconolactone | 5 | 28 |
| Gluconolactone | 10 | 1 |

Table 3. *Effect of sulphydryl reactants on isoamylase activity*

The composition of the digests was as in Table 2, except that the final volume was 25 ml. Samples (2·5 ml.) were removed initially and after (*a*) 2·5 hr. and (*b*) 22 hr. for analysis.

| Conditions | Concn. (mM) | Activity (increase in extinction, %) (*a*) | (*b*) |
|---|---|---|---|
| Control | — | 22 | 34 |
| Iodoacetate | 10 | 17 | 25 |
| Phenylmercuric acetate | 0·01 | 10 | 20 |
| *p*-Chloromercuribenzoate | 0·01 | 9 | 24 |

glucose. Part of the solution (13 ml.) was then incubated with isoamylase (60 mg.) and buffer (4 ml.). After 24 hr. at 20° the reducing power had not increased. It is concluded that isoamylase has no action on α-(1 → 3)-linkages.

*Action on β-(1 → 6)-glucosidic linkages.* Pustulan, an insoluble linear polysaccharide composed of β-(1 → 6)-glucosidic linkages (Lindberg & McPherson, 1954) was partially hydrolysed (2 %, w/v) in N-sulphuric acid at 98° for 135 min.). After cooling, neutralization (barium carbonate), and filtration, the filtrate was concentrated *in vacuo* at 35°, and shown by paper chromatography to contain a regular series of oligosaccharides, together with glucose. Part of the concentrate (≡ 40 mg. of pustulan) was incubated with isoamylase (60 mg.) and buffer (3 ml.) in a final volume of 25 ml. No change in reducing power was observed during incubation at 20° for 24 hr. Hence, isoamylase has no action on β-(1 → 6)-glucosaccharides.

*Action on glycogen.* Incubation of isoamylase with glycogen samples from a variety of biological sources caused a marked increase in β-amylolysis limit ranging from 10 to 32 %. The results are shown in Table 4. Isoamylolysis is considered to be complete for two reasons: first, the increase in the β-amylolysis limit was the same after incubation with isoamylase for 24, 48 or 72 hr.; secondly, addition of glycogen to a 72 hr. digest of isoamylase and glycogen caused an increase in β-amylolysis limit from 44 to 51 %, thus showing that active isoamylase was still present.

The product of the action of isoamylase on a sample of oyster glycogen was then examined in detail; for convenience this will be referred to as 'isodextrin' but this does not necessarily indicate a limit-dextrin configuration.

Table 4. *Effect of isoamylase on the β-amylolysis limit of polysaccharides*

β-Amylolysis limits were measured before (*b*) and after (*a*) treatment with isoamylase at pH 5·9 and 18° for 18–24 hr.

| | Average chain length* | β-Amylolysis limit (%) | |
|---|---|---|---|
| | | (*b*) | (*a*) |
| Glycogen sample | | | |
| *Helix pomatia* | 7 | 37 | 60 |
| Human liver | 14 | 46 | 60 |
| Human kidney | 14 | 46 | 59 |
| Oyster | 10 | 40 | 65 |
| Rabbit liver (IV) | 13 | 46 | 78 |
| (V) | 12 | 52 | 76 |
| (X) | 12 | 49 | 59 |
| Synthetic† | 14 | 47 | 75 |
| *Trichomonas foetus* | 15 | 60 | 80 |
| Yeast (brewer's) | 13 | 44 | 68 |
| Commercial | 13 | 45 | 65 |
| Amylopectin sample | | | |
| *Dunaliella bioculata* | 15–16 | 61 | 76 |
| Potato I | 22 | 53 | 80 |
| II | 21 | 61 | 77 |
| Protozoal | 22 | 63 | 80 |
| Synthetic‡ | — | 57 | 82 |
| Waxy-maize starch | 20 | 50 | 66 |
| Waxy-maize starch β-dextrin | 10 | 0 | 71 |
| Waxy-sorghum starch | 22 | 52 | 76 |

* Determined by periodate oxidation.

† Prepared by the action of yeast branching enzyme on amylopectin (Gunja, Manners & Khin Maung, 1960).

‡ Prepared by the action of yeast branching enzyme on amylose (Gunja *et al.* 1960).

*Preparation and properties of glycogen isodextrin*

Glycogen (1 g.) was incubated with isoamylase (fraction 2; 100 mg.), buffer (pH 5·9; 10 ml.) and water (30 ml.) at 21° for 48 hr. The enzyme was then inactivated by heating (15 min. at 98°). After removal of coagulated protein by filtration (G. 3 filter), the solution was dialysed against distilled water at room temperature for 48 hr., and the isodextrin isolated by freeze-drying. Yield, 0·85 g.

Isodextrin (147 mg.) and glycogen (255 mg.) were oxidized with a suspension of potassium metaperiodate (110 ml.) at room temperature, as described by Gunja *et al.* (1960). The production of formic acid was constant after 10 days and amounted to 2·33 and 2·80 mg. respectively. These figures correspond to average chain lengths of 12·0 and 10·0 glucose residues. The isodextrin had β-amylolysis limits of 50 and 58 % before and after treatment with isoamylase, and the iodine complex showed maximum absorption at 435–440 mμ. For the original glycogen, the corresponding figures were 40 % and 420 mμ.

*Stepwise action of isoamylase and β-amylase on glycogen*

Yeast glycogen (25 mg.) was incubated with isoamylase (40 mg.) in a total volume of 13 ml. at pH 5·9 and 20° for 24 hr. After heat inactivation, β-amylase was added, and, after 24 hr., the β-amylolysis limit was 67 %. The β-amylase was then inactivated by heating, and isoamylase solution (40 mg. in 1 ml. of water) added. This treatment caused only a small increase in the reducing power of the digest (from 17·6 to 18·4 mg. of apparent maltose) but the β-amylolysis limit of the polysaccharide increased to 81 %. Removal of the outer chains of the glycogen isodextrin had thus facilitated further isoamylolysis.

*Action of isoamylase on amylopectin and amylopectin β-dextrin*

The β-amylolysis limits of various amylopectin samples determined before and after incubation with isoamylase are shown in Table 4.

Amylopectin isodextrin was prepared from potato amylopectin (1 g.) by a method similar to that described for glycogen isodextrin, and, as before, the product was not a limit-dextrin. Yield, 0·6 g. Amylopectin isodextrin had $\beta$-amylolysis limits of 45 and 80 % before and after treatment with isoamylase, and the iodine complex showed maximum absorption at 550 m$\mu$. The limiting viscosity numbers for potato amylopectin and the isodextrin were 205 and 41 respectively.

Incubation of amylopectin $\beta$-dextrin with isoamylase (fraction 2) failed to produce the expected increase in iodine-staining power (see Discussion). In one experiment, the extinctions (at 540 m$\mu$) of the polysaccharide–iodine complexes after isoamylolysis for 0·2, 1, 2 and 24 hr. were respectively 0·11, 0·12, 0·12 and 0·11. The same enzyme preparation increased the extinction of the iodine complex of a glycogen from 0·055 to 0·079 within 1 hr. However, the $\beta$-amylolysis limit of amylopectin $\beta$-dextrin always increased.

With amylopectin $\beta$-dextrin and an acid-treated isoamylase preparation, maltose and maltotriose were produced (paper chromatography). This provides a further indication that the A-chain stubs in an amylopectin $\beta$-limit dextrin contain two or three glucose residues (cf. Peat *et al.* 1952).

### *Simultaneous action of isoamylase and β-amylase on polysaccharides*

Since isoamylase causes a greater increase in the $\beta$-amylolysis limit of amylopectin $\beta$-dextrin than of amylopectin (Table 4), it was possible that isoamylolysis was being hindered by the presence of long outer chains. The simultaneous action of iso- and $\beta$-amylase on various polysaccharides was therefore examined, as, under these conditions, exterior chains would be reduced to stubs of only two to three glucose residues. Polysaccharides (15–25 mg.), isoamylase (40 mg.), $\beta$-amylase (1000 units), buffer (4 ml.) and water to 25 ml. were incubated at 20° for 24 hr. The following conversions (%) into maltose were obtained: potato amylopectin, 84; waxy-maize starch, 83; oyster glycogen, 95; synthetic glycogen (see Gunja *et al.* 1960), 90. In a control digest containing maltose, no increase in reducing power was observed. It follows that very extensive degradation of the polysaccharides had occurred.

*Action on isomaltose.* Paper-chromatographic analysis showed that fraction 2 slowly hydrolysed isomaltose to glucose (Manners & Khin Maung, 1955) and it was concluded that isoamylase could attack terminal $\alpha$-(1 $\rightarrow$ 6)-glucosidic linkages. However, more recent evidence suggests that isoamylase has no action on isomaltose.

Isomaltose (7·7 mg.), buffer (5 ml.), gluconolactone (4·5 % solution; 0, 0·5 or 1·0 ml.) and isoamylase (25 mg.) were incubated in a total volume of 25 ml. at 20°. After 24 hr., the apparent increases in maltose equivalents were 1·2, 1·0 and 0·0 respectively, and at 72 hr., 1·9, 1·8 and 0·0. The isomaltase activity is therefore completely inhibited by 10 mM-gluconolactone but is little altered in the presence of 5 mM-inhibitor, whereas isoamylase activity is reduced by 56 % under these conditions.

The non-identity of yeast isomaltase and isoamylase has been confirmed by the separation of these two activities by chromatography on alumina or continuous paper electrophoresis (Gunja, 1959) and independently by Kobayashi (1958) using starch-gel electrophoresis.

*Action on α-dextrins.* Maltodextrin (10 mg.), isoamylase (21 mg.), buffer (pH 5·9; 1 ml.) and water to 5 ml. were incubated at 20° for 24 hr. After heating and cooling, the $\beta$-amylolysis limit was determined. The result, 94 %, may be compared with that of the original maltodextrin, 67 %. Most of the $\alpha$-(1 $\rightarrow$ 6)-linkages in the maltodextrin were therefore hydrolysed.

## DISCUSSION

The present study shows that extracts of brewer's yeast contain a carbohydrase, isoamylase, which hydrolyses a proportion of the $\alpha$-(1 $\rightarrow$ 6)-glucosidic inter-chain linkages in both glycogen and amylopectin. In this respect it is similar to the debranching enzyme in black-koji mould (Ueda, 1957), but clearly differs from the corresponding plant and animal debranching enzymes, R-enzyme and amylo-(1 $\rightarrow$ 6)-glucosidase.

Although extensive purification of isoamylase has not been attempted, acetone fractionation of yeast extracts has consistently given preparations with activity sufficient to enable the action pattern and some of the properties of the enzyme to be determined. For example, isoamylase action is optimum at about pH 6 and 25° [cf. the properties of amylosynthease reported by Nishimura & Minagawa (1931)] and, unlike $\beta$-amylase, does not need SH groups for activity.

The available evidence shows that isoamylolysis of amylopectin and glycogen is incomplete since some 10–20 % is resistant to the successive actions of isoamylase and $\beta$-amylase (see Table 4). The inability of isoamylase to hydrolyse all the inter-chain linkages appears to be due to steric hindrance caused by the exterior portions of the B-chains in the interior of the molecules. This is shown by the greater degradation caused by the simultaneous action of these two enzymes. This situation is similar to that observed during R-enzyme action on amylopectin and amylopectin $\beta$-dextrin (Hob-

son *et al.* 1951); these polysaccharides then had $\beta$-amylolysis limits of 74 and 73 % respectively, the latter corresponding to 87 % degradation of the original amylopectin.

Evidence that isoamylase removes a large proportion of A-chains is shown by the end-group assay of glycogen isodextrin. Assuming the presence of equal numbers of A- and B-chains in a glycogen of chain length 10 and $\beta$-amylolysis limit 40 %, the average lengths of the A- and B-chains are 6·5 and 13·5 glucose residues respectively. Hydrolysis of A-chains, and subsequent removal by dialysis, will therefore cause an overall increase in average chain length; experimentally, the increase was two glucose residues. This evidence is also confirmed by comparison of the observed $\beta$-amylolysis limit after isoamylase treatment with that calculated on the assumption that all the A-chains have been removed. For yeast glycogen the figures are 68 and 68 % respectively, and for potato amylopectin, 80 and 75 %.

The action of isoamylase on amylopectin produces a marked decrease (80 %) in viscosity, and hence in molecular weight, and an increase in $\beta$-amylolysis limit. The general conclusions of Maruo & Kobayashi (1951) are therefore substantiated, and a similarity with the action of R-enzyme on amylopectin is established (Hobson *et al.* 1951). However, isoamylolysis did not increase the iodine-staining power, as expected; it is probable that the presence of branching-enzyme impurity would effectively mask such changes (cf. Gunja *et al.* 1960), although, since yeast branching enzyme has no action on glycogen, measurement of activity towards the latter substrate is not affected. In the experiments of Kobayashi (1956), the activity of isoamylase could be followed by measurement of the increase in iodine coloration (at 620 m$\mu$) of glutinous rice starch.

Since isoamylase appears to have no action on $\alpha$-(1 $\rightarrow$ 3)-, $\alpha$-(1 $\rightarrow$ 4)- or $\beta$-(1 $\rightarrow$ 6)-glucosidic linkages, it provides a useful tool for the structural analysis of starch-type polysaccharides. Examples of its use (see Table 4) include the characterization of inter-chain linkages in protozoal and algal starches, which are not available in the quantity required for examination by methylation or periodate oxidation techniques. Nevertheless, the conclusion on specificity must be considered with caution. The failure of a carbohydrase to attack a particular linkage in an oligosaccharide does not necessarily imply that the same linkage in a polysaccharide would also be resistant. Thus R-enzyme will not hydrolyse isomaltose, but acts on amylopectin. Substrates containing for example both $\beta$-(1 $\rightarrow$ 6)- and $\alpha$-(1 $\rightarrow$ 4)-glucosidic linkages are not available for examination. We have therefore concluded that it is unlikely that isoamylase has any action on linkages other than the non-terminal $\alpha$-(1 $\rightarrow$ 6)-glucosidic type as present in starch-type polysaccharides.

The incomplete degradation of waxy-maize starch and potato amylopectin by the combined action of iso- and $\beta$-amylase has structural significance. This may indicate the presence of linkages other than the $\alpha$-(1 $\rightarrow$ 4)- or $\alpha$-(1 $\rightarrow$ 6)-glucosidic type. Residues of glucose 6-phosphate are possible resistant structures (cf. Posternak, 1951). Alternatively, the presence of a small proportion of adjacent $\alpha$-(1 $\rightarrow$ 6)-linked glucose residues [which give rise to doubly and triply linked $\alpha$-dextrins on $\alpha$-amylolysis (see Roberts & Whelan, 1960)] may prevent iso- and $\beta$-amylolysis.

The presence of a debranching enzyme in yeast extracts has been shown independently by Hopkins (1955, 1958) and Hopkins & Kulka (1957*a*, *b*) during a study of dextrin fermentation. With amylopectin $\beta$-dextrin as substrate, and in the presence of $\beta$-amylase, various yeast preparations caused a decrease in iodine-staining power. Enzyme action was, however, incomplete, about two-thirds of the $\beta$-dextrin being resistant. In the absence of $\beta$-amylase, the iodine-staining power increased. Possible activity towards glycogen was not reported. Some differences in the debranching enzymes from *Saccharomyces diastaticus* and *S. cerevisiae* were noted; thus the former activity was the more stable at pH 4·6.

The ability of fraction 2 to cause an increase in the $\beta$-amylolysis limit of maltodextrin would suggest that isoamylase also possesses 'limit-dextrinase' activity. MacWilliam & Harris (1959) have shown that preparations of R-enzyme that attack the $\alpha$-(1 $\rightarrow$ 6)-linkages in both amylopectin and $\alpha$-limit dextrin can be separated into two specific enzymes, one acting only on amylopectin and the other only on $\alpha$-limit dextrin. The possibility that the yeast limit-dextrinase activity is caused by a separate carbohydrase is therefore now being examined.

In all the above experimental work, a brewer's yeast was used. However, extraction of a baker's yeast with bicarbonate followed by fractionation with acetone has also given preparations showing isoamylase activity. For example, the protein precipitated by 0–20, 20–33 and 33–42 % concentration of acetone increased the $\beta$-amylolysis limit of glycogen by 5, 24 and 23 % respectively (Gunja, 1959).

## SUMMARY

1. Extracts of brewer's yeast contain a debranching enzyme, isoamylase, which may be isolated by acetone fractionation at $-7°$.

2. Enzyme action on glycogen is optimum at about pH 6 and 25°, is inhibited by borate (10 mM)

and mercuric chloride (0·1 mM) but not by sulphydryl reactants.

3. The reaction is characterized by a marked increase in iodine-staining power and β-amylolysis limit but α-(1 → 4)-glucosidic linkages in glycogen are not attacked. Enzyme action is incomplete, only the outer α-(1 → 6)-glucosidic linkages being hydrolysed.

4. Isoamylase differs from the plant and animal debranching enzymes [R-enzyme and amylo-(1 → 6)-glucosidase] since both amylopectin and glycogen are substrates.

5. Purified isoamylase has no action on maltose, isomaltose or maltotriose.

6. Isoamylase is also present in baker's yeast.

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# The Metabolism of [4-$^{14}$C]Progesterone in the Cat: Biliary and Urinary Excretion of Conjugated Metabolites

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Marrian (1949, 1954) stressed the need for a systematic study of progesterone metabolism in order to account for the large proportion of that hormone which is not excreted in the urine, by man and some animals, as pregnanediol (5β-pregnane-3α:20α-diol) and closely related substances. The availability of steroid hormones labelled with $^{14}$C and $^{3}$H now permits such systematic studies to be carried out, and some investigations with labelled progesterone have been reported. When [21-$^{14}$C]-

progesterone was administered to rats and mice the major part of the excreted radioactivity was present in the bile or faeces, but no attempt was made to characterize the metabolites (Riegel, Hartop & Kittinger, 1950; Grady, Elliott, Doisy, Bocklage & Doisy, 1952; Shen, Elliott, Doisy & Doisy, 1954). In contrast, after administration of [4-$^{14}$C]progesterone to human subjects, more than 50% of the excreted radioactivity was in the urine and up to 30% in the bile or faeces (Davis & Plotz, 1957; Sandberg & Slaunwhite, 1958). A post-menopausal woman investigated by Wiest, Fujimoto & Sandberg (1958) excreted a larger proportion of radioactive metabolites in the bile than in the urine: pregnanediol, pregnanolone (3$\alpha$-hydroxy-5$\beta$-pregnan-20-one) and more 'polar' material were found in the $\beta$-glucuronidase-hydrolysed fractions of the bile and urine. Pearlman (1957) studied the metabolism of [16-$^{3}$H]progesterone in human females but confined himself to investigating metabolites present in neutral extracts after hot-acid hydrolysis of the urine. In the light of the results reported above it was felt that further investigation of progesterone metabolism *in vivo* might prove of value.

Because of the difficulties and risks associated with the use of labelled steroids in human subjects, and because of the difference between rodents and man described above, it was decided to investigate the metabolism of [4-$^{14}$C]progesterone in cats. These animals are of sufficient size to make the necessary surgical procedures relatively easy, and to permit the administration of a reasonably large dose of the steroid. In this investigation male cats were used to eliminate any possible effects on progesterone metabolism of the ovarian status of the animals. Labelled progesterone has been administered intravenously to three pairs of anaesthetized animals: the first pair received a single rapid injection in 10 sec. and in the second and third pairs the steroid was given as a continuous infusion over 1 and 3 hr. respectively. Bile and urine were collected and treated by standard hydrolytic methods. The neutral, ether-extractable radioactivity in each fraction was determined. A preliminary account of part of this work has been published (Taylor & Scratcherd, 1960).

## MATERIALS AND METHODS

[4-$^{14}$C]*Progesterone.* [4-$^{14}$C]Progesterone (20·8 mc/mmole) was obtained in benzene solution from The Radiochemical Centre, Amersham, Bucks. Approximately ten times the weight of unlabelled progesterone, which had been crystallized twice from hexane and once from aqueous ethanol and had m.p. 121–121·5°, was added. The benzene was evaporated in a stream of air at 45° and the progesterone was crystallized once from hexane. The crystals were stored in the dark at −15° and, although a slightly yellowish colour developed after a few days, no detectable deterioration occurred in the several weeks of storage. Progesterone solutions were prepared by dissolving the steroid in 0·5 ml. of warm ethanol and adding the required amount of 0·154 M-NaCl immediately before injection (0·5 ml. for rapid single injections, 25 ml. for the more prolonged infusions).

*Operative procedure.* Male cats, which had been starved of solid food overnight but allowed access to water and milk, were anaesthetized by intravenous injection of a mixture of 2% (w/v) of chloralose (75 mg./kg.) and 20% (w/v) of urethan (450 mg./kg.) after preliminary induction with ether. The splanchnic nerves were cut extraperitoneally. To collect bile a glass cannula (5 cm. long, 0·2 cm. bore) was inserted through the fundus of the gall bladder. One end of the cannula was covered with soft rubber and was placed as close as possible to the opening of the cystic duct. The common bile duct was tied off. Urine was collected by passing a cannula through the apex of the bladder, the cannula being tied in place so that its tip was adjacent to the openings of the ureters. All solutions were injected into a cannula in the saphenous vein. Rectal temperature was maintained at 38·5°. After the operation the animal was left for 2 hr. before injection of the steroid to ensure that the bile and urine flows were steady. Generally the early bile and urine samples were contaminated with small amounts of blood, but no detectable contamination occurred during the collection period proper.

At the end of each experiment the liver and kidneys of each animal were removed. No abnormality was detected on gross or histological examination.

Injections of radioactive solutions were made through a three-way adapter which allowed the original syringe to be rinsed out with 0·9% NaCl solution without removing it from the cannula. Prolonged infusions were carried out by connecting the syringe to a calibrated, electrically driven plunger which gave a constant rate of infusion.

*Treatment of bile and urine samples.* Immediately after collection the bile and urine samples were weighed and then dried to constant weight in an evacuated desiccator containing conc. $H_2SO_4$. The dry residues were dissolved in a known volume of water. Portions of the solutions were plated in duplicate and the total corrected radioactivity was determined. Volumes of the solutions equivalent to 5000–10 000 counts/min. were then subjected to extractions and hydrolyses to provide the following fractions:

(1) Non-conjugated. Radioactivity extractable before any form of hydrolysis.

(2) Glucuronides. After the initial extraction the sample was taken to pH 4–5 with 2 N-acetic acid, 0·5 vol. of 0·2 M-acetate buffer, pH 4·5, containing 5000 units of $\beta$-glucuronidase, and 0·1 vol. of 0·2 M-$KH_2PO_4$ were added, and the mixture was incubated at 45° for 18 hr. and then extracted.

(3) Cold-acid-hydrolysed metabolites. The enzyme-hydrolysed solution was then made 2 N with respect to $H_2SO_4$ by the addition of 24 N-$H_2SO_4$. The acid was added drop by drop while the tubes were immersed in ice–water. A volume of wet ether equal to the total volume of aqueous solution was added, and the tubes were left at room temperature with occasional shaking (Burstein & Lieberman, 1958). After 72 hr. the ether was removed and the aqueous phase was extracted twice more with ether.

(4) Hot-acid-hydrolysed metabolites. The acidified aqueous solution remaining after the previous hydrolysis

was now less than 2 N with respect to $H_2SO_4$ because of the addition of the alkali and water washings of the ether extract, but the pH was still less than 1. Ether dissolved in the water was removed by placing the tubes in a water bath at 45° and playing a stream of air over the surface of the liquid. The tubes were then heated in a boiling-water bath for 30 min., cooled and extracted.

(5) Water-soluble metabolites. After all the above methods of hydrolysis the aqueous solution was neutralized with 5 N-NaOH. When sufficient radioactivity was present portions of the solution could be plated directly and self-absorption corrections applied. In most cases, however, the concentration of radioactivity was low, and the $^{14}C$ content was therefore determined by the differential counting method of Heard, Thompson & Yates (1952).

*Extraction procedure.* AnalaR ether was washed with acidified $FeSO_4$ and then with water until neutral, dried over anhydrous $Na_2SO_4$ and distilled. Wet ether used for extractions was prepared by saturating the distilled ether with water.

Aqueous solutions were extracted three times with 2 vol. of wet ether. The combined ether extracts were washed twice with 0·1 vol. of 0·1 N-NaOH and then with 0·1 vol. of water until neutral, the alkali and water washings being added to the original aqueous solution. The washed extracts were dried over anhydrous $Na_2SO_4$ for at least 2 hr. and then filtered. The $Na_2SO_4$ was washed three times with small amounts of dry ether, and the combined washings and filtrates were evaporated to dryness on a water bath at 45°. The dry residues were dissolved in chloroform–methanol (1:1, v/v), the volume used depending on the amount of radioactivity in the extracts. Portions of the solution were plated in duplicate and their radioactivities determined.

*β-Glucuronidase.* Powder B obtained from acetone-dried limpet viscera as described by Dodgson & Spencer (1953) was used as a source of β-glucuronidase. It contained $10^6$ Fishman units/g. Since this preparation also contains sulphatases, hydrolyses were carried out in the presence of 0·02 M-$KH_2PO_4$, a potent inhibitor of these enzymes (Dodgson & Spencer, 1953; Roy, 1956). The dry powder was homogenized in buffer solution before addition to incubation vessels. In β-glucuronidase-inhibition experiments 0·16 M-potassium hydrogen saccharate was boiled for 30 min., cooled, taken to pH 4·5 and added to incubation vessels to give a final concentration of 0·03 M (Levvy, 1952).

*Assay of radioactivity.* This was carried out in a windowless gas-flow proportional counter (SC-16P; Tracerlab Inc., Boston, Mass., U.S.A.); argon–methane (90:10, v/v) was supplied by British Oxygen Co. Ltd. Samples were plated on duralumin planchets (1 in. diam, and scoured with concentric grooves to ensure uniform distribution of material) and dried under an infrared lamp. All assays of extracts were performed at infinite thinness (<100 μg. of total solids/planchet); counts of urine and bile were corrected to infinite thinness by reference to appropriate self-absorption correction curves. All samples were counted for at least 1000 counts/min., so that counting errors did not exceed 5%. Day-to-day instrumentation errors were corrected by the use of poly[$^{14}C$]methyl methacrylate reference standards (The Radiochemical Centre). Under the above conditions counting efficiency was estimated to be 45–48%. Throughout this paper quantities of radioactivity are expressed as counts/min. actually determined.

## RESULTS

Table 1 shows the excretion of radioactivity in the bile of cats after a single rapid injection (Table 1) or after 1 or 3 hr. infusion of [4-$^{14}C$]progesterone. The volumes of bile collected and the concentrations of total solids are also included to show that biliary function was maintained throughout the collection period. There was wide variation in the volume of bile produced by different animals, ranging from 0·1 (cat no. 5) to 3·9 ml./hr. (cat no. 2). In general, however, there was a definite decrease in bile volume with time, presumably due, at least in part, to the absence of the choleretic activity of bile salts that, under normal conditions, would have been returned to the liver via the enterohepatic circulation. Since all samples collected were golden-brown in colour it can be concluded that essentially liver bile was collected, and this is borne out by the concentrations of solids in the samples collected. A sample of gall-bladder bile obtained from eight cats used for other purposes was very dark-green and contained 11·2 g. of solids/100 ml. Only the bile of cat no. 6 approached this value and it should be noted that this animal excreted only a small volume of bile.

Table 1 shows that, after a single dose of progesterone administered in 10 sec., excretion of metabolites was rapid, over 60% of the dose being recovered after 4 hr. Excretion of radioactivity reached a maximum during the first or second hour after injection of the steroid and declined thereafter. A similar trend in excretion of radioactivity can be seen from the results of the experiments in which the steroid was infused over 1 or 3 hr., but in these experiments the trends are less definite because of the longer period over which the steroid was given. The amount of radioactivity recovered after infusion was considerably less (26·2–55·3%) than after a single injection (65·6–67·1%). This was not due to the length of the collection period after the end of the administration of the steroid, since bile was collected from cat no. 5 for 5 hr. after the end of the 1 hr. perfusion and a total of 35·2% was recovered, whereas, on the other hand, after a single dose of progesterone cats nos. 1 and 2 had excreted approximately 60% of the dose in less than 5 hr. These findings suggest that a single rapid dose of steroid is handled differently in the body from a similar dose administered over a longer period. Table 1 also shows the distribution of excreted neutral radioactivity after hydrolysis, the amount of radioactivity remaining water-soluble and the percentage recovery of radioactivity after application of the successive hydrolytic methods.

Table 2 gives the corresponding results for the urines obtained from the same six cats. As with

Table 1. *Excretion of radioactivity in bile of male cats after intravenous injection of [4-$^{14}C$]progesterone*

A single dose was given to cats 1 and 2; cats 3 and 4, and 5 and 6 were dosed by continuous infusion for 1 and 3 hr. respectively.

Table 1. *Excretion of radioactivity in bile of male cats after intravenous injection of* [4-$^{14}$C]*progesterone*

A single dose was given to cats 1 and 2: cats 3 and 4, and 5 and 6, were dosed by continuous infusion for 3 and 1 hr. respectively.

| Cat no. | Wt. (kg.) | Dose of steroid (mg.) | Dose of steroid ($10^{-6}$ × counts/min.) | Collection period (hr.) | Bile vol. (ml.) | Total solids (mg.) | Total solids (g./100 ml.) | Bile ($10^{-6}$ × total counts/min.) | Bile (% of dose) | Distribution of radioactivity (% of total counts in sample) after successive hydrolyses: Neutral, ether-soluble radioactivity | | | | Total recovery after all hydrolyses (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | Non-conjugated | Glucuronide | Cold-acid hydrolysed | Hot-acid hydrolysed | Water-soluble |
| 1 | 3·7 | 0·922 | 10·00 | 1·0 | 2·9 | 73 | 2·5 | 2·551 | 25·5 | 0·5 | 25·6 | 12·2 | 11·1 | 46·3 | 95·7 |
| | | | | 2·0 | 2·6 | 62 | 2·4 | 1·634 | 16·3 | | | | | | |
| | | | | 3·0 | 2·5 | 57 | 2·3 | 1·077 | 10·8 | 0·5 | 10·3 | 22·0 | 10·7 | 47·1 | 90·6 |
| | | | | 4·0 | 2·1 | 45 | 2·1 | 0·637 | 6·4 | | | | | | |
| | | | | 5·0 | 1·7 | 38 | 2·2 | 0·544 | 5·4 | 0·3 | 5·9 | 32·2 | 6·9 | 50·2 | 95·5 |
| | | | | 5·5 | 1·4 | 32 | 2·3 | 0·272 | 2·7 | | | | | | |
| | | | | | | | | Total | 67·1 | | | | | | |
| 2 | 3·7 | 1·390 | 9·56 | 1·0 | 1·4 | 55 | 3·9 | 1·813 | 18·7 | 0·5 | 40·1 | 9·4 | 13·3 | 24·7 | 88·0 |
| | | | | 2·0 | 1·3 | 39 | 3·0 | 2·307 | 24·0 | 0·6 | 20·9 | 13·2 | 20·0 | 34·3 | 89·0 |
| | | | | 3·0 | 0·7 | 27 | 2·9 | 1·098 | 11·5 | 0·3 | 13·5 | 14·9 | 18·4 | 42·6 | 89·7 |
| | | | | 4·0 | 1·0 | 39 | 3·9 | 1·096 | 11·4 | 0·5 | 10·8 | 17·9 | 20·5 | 47·9 | 97·6 |
| | | | | | | | | Total | 65·6 | | | | | | |
| 3 | 3·0 | 2·580 | 30·30 | 4·5 | 8·6 | 260 | 3·0 | 16·77 | 55·3 | 0·5 | 24·5 | 26·4 | 4·5 | 38·2 | 94·1 |
| 4 | 4·1 | 2·592 | 31·34 | 1·0 | 3·5 | 98 | 2·8 | 0·667 | 2·2 | 1·3 | 15·7 | 31·8 | 25·6 | 24·0 | 98·4 |
| | | | | 2·0 | 3·6 | 72 | 2·0 | 1·820 | 6·1 | 0·7 | 10·6 | 25·5 | 25·5 | 25·8 | 88·4 |
| | | | | 3·0 | 3·7 | 71 | 1·9 | 1·942 | 6·5 | 1·0 | 8·8 | 23·8 | 28·6 | 27·4 | 89·7 |
| | | | | 4·0 | 3·8 | 67 | 1·8 | 2·194 | 7·3 | 0·9 | 8·6 | 24·2 | 27·0 | 33·7 | 94·4 |
| | | | | 5·0 | 3·3 | 63 | 1·9 | 1·470 | 4·9 | 0·7 | 6·3 | 21·8 | 26·1 | 35·2 | 90·1 |
| | | | | 6·0 | 2·7 | 54 | 1·9 | 1·090 | 3·6 | 0·7 | 3·7 | 18·6 | 27·0 | 43·7 | 93·7 |
| | | | | | | | | Total | 30·6 | | | | | | |
| 5 | 2·8 | 1·251 | 10·125 | 1·0 | 0·6 | 30 | 5·0 | 0·058 | 0·6 | 0 | 43·1 | 19·0 | 37·9 | 3·4 | 103·7 |
| | | | | 2·0 | 0·6 | 27 | 4·5 | 0·752 | 7·5 | 0·5 | 36·6 | 24·1 | 24·7 | 7·4 | 93·3 |
| | | | | 3·0 | 0·6 | 22 | 3·6 | 1·026 | 10·3 | 0·4 | 18·4 | 30·3 | 34·0 | 5·8 | 88·9 |
| | | | | 4·0 | 0·5 | 18 | 3·6 | 0·828 | 8·3 | 0·4 | 10·5 | 28·9 | 33·0 | 17·8 | 90·6 |
| | | | | 5·0 | 0·1 | 6 | 6·0 | 0·229 | 2·3 | 0·1 | 9·6 | 34·1 | 35·8 | 13·0 | 92·6 |
| | | | | 6·0 | 0·3 | 16 | 5·3 | 0·618 | 6·2 | 0·2 | 9·2 | 38·3 | 26·9 | 22·0 | 96·6 |
| | | | | | | | | Total | 35·2 | | | | | | |
| 6 | 4·0 | 2·055 | 8·756 | 1·0 | 0·3 | 32·5 | 10·0 | 0·005 | 0·1 | 0 | 28·0 | 40·0 | 29·5 | 0 | 97·5 |
| | | | | 2·0 | 0·5 | 39·9 | 7·5 | 0·460 | 5·2 | 0·4 | 22·2 | 18·3 | 10·2 | 42·5 | 93·6 |
| | | | | 3·0 | 0·7 | 43·6 | 5·8 | 1·287 | 14·7 | 0·3 | 15·8 | 22·5 | 10·4 | 50·0 | 99·0 |
| | | | | 4·0 | 0·3 | 19·0 | 5·5 | 0·543 | 6·2 | 0·4 | 10·3 | 10·8 | 9·2 | 58·0 | 88·7 |
| | | | | | | | | Total | 26·2 | | | | | | |

Table 2. *Excretion of radioactivity in urine of the cats of Table 1*

| Cat no. | Collection period (hr.) | Urine vol. (ml.) | Urine: Total solids (g.) | Urine: Total solids (g./100 ml.) | Urine: ($10^{-4}$ × total counts/min.) | Urine: (% of dose) | Distribution of radioactivity (% of total counts in sample) after successive hydrolyses: Neutral, ether-soluble radioactivity: Non-conjugated | Glucuronide | Cold-acid hydrolysed | Hot-acid hydrolysed | Water-soluble | Total recovery after all hydrolyses (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 5·5 | 15·6 | 2·43 | 15·6 | 4·70 | 0·5 | 1·3 | 5·0 | 6·3 | 11·7 | 70·2 | 94·5 |
| 2 | 4·0 | 4·8 | 1·28 | 26·9 | 4·60 | 0·5 | 1·0 | 5·8 | 8·2 | 10·2 | 69·6 | 94·8 |
| 3 | 4·5 | 20·0 | 1·47 | 7·3 | 24·00 | 0·8 | 3·7 | 7·4 | 11·7 | 1·2 | 66·7 | 90·7 |
| 4 | 6·0 | 11·0 | 2·80 | 25·4 | 8·55 | 0·28 | 5·4 | 2·7 | 3·2 | 4·1 | 77·2 | 92·6 |
| 5 | 2·0 | 21·8 | 1·11 | 5·1 | 2·20 | 0·22 | 1·4 | 5·5 | 6·2 | 7·5 | 74·3 | 94·8 |
| | 4·0 | 16·9 | 0·86 | 5·1 | 2·40 | 0·24 | 1·5 | 3·3 | 4·5 | 8·4 | 73·4 | 91·1 |
| | 6·0 | 7·4 | 0·56 | 7·6 | 1·87 | 0·19 | 1·3 | 3·1 | 4·4 | 6·6 | 77·6 | 93·0 |
| | | | | | Total | 0·65 | | | | | | |
| 6 | 2·0 | 4·0 | 0·56 | 13·9 | 0·764 | 0·09 | 1·3 | 5·5 | 6·0 | 4·3 | 80·0 | 97·1 |
| | 4·0 | 4·9 | 0·82 | 16·7 | 2·730 | 0·31 | 1·0 | 1·5 | 4·8 | 6·3 | 70·0 | 83·6 |
| | | | | | Total | 0·40 | | | | | | |

bile there was wide variation among animals in the volume of urine excreted, ranging from 4·8 ml. in 4 hr. (cat no. 2) to 47·1 ml. in 6 hr. (cat no. 5), and also in the total urinary solids, 5·1 % (cat no. 5) to 26·9 % (cat no. 2). This variation probably reflects the degree of hydration of the animals at the time of the experiments. However, there appears to be no correlation between the volumes of bile and urine produced by an animal: e.g. cat no. 5 produced a large volume of relatively dilute urine (Table 2) but only a small volume of concentrated bile (Table 1), whereas cat no. 2 excreted the smallest volume of the most highly concentrated urine (Table 2) and a bile of intermediate volume and concentration (Table 1).

The amount of radioactivity excreted in the urine ranged from 0·28 to 0·8 % of the dose and appeared to be independent of the method of administration of the steroid. Table 2 also shows the amounts of neutral radioactivity recovered after hydrolysis, the amount of radioactivity remaining water-soluble after hydrolysis and the percentage of radioactivity recovered from the sample analysed after the successive hydrolyses.

Table 3 shows the results of experiments carried out to investigate the effect of boiled saccharate solution and phosphate on the release of ether-extractable, neutral radioactivity by incubation of bile and urine with limpet powder. It can be seen that 0·03 M-saccharate almost completely inhibited the release and 0·02 M-phosphate had no effect.

Table 3. *Effect of boiled saccharate and potassium dihydrogen phosphate on liberation of radioactivity from bile and urine by limpet powder*

Each vessel contained 0·2 ml. of bile or 1·0 ml. of urine, 400 $\mu$g. of limpet powder and 0·05 M-acetate buffer, pH 4·5, in a final vol. of 3·5 ml. Final concentrations of inhibitors, when added, were 0·03 M-saccharate and 0·02 M-$KH_2PO_4$. Incubations, which were carried out in duplicate, were for 2 hr. at 37°.

| Additions to incubation medium | Neutral counts/min. extracted: Bile | Urine |
|---|---|---|
| (Boiled enzyme) | 106 | 47 |
| | 94 | 30 |
| Nil | 26 100 | 1040 |
| | 26 000 | 1010 |
| Saccharate | 182 | 52 |
| | 162 | 45 |
| Saccharate + $KH_2PO_4$ | 141 | 43 |
| | 128 | 37 |
| $KH_2PO_4$ | 27 000 | 1050 |
| | 26 000 | 1070 |

## DISCUSSION

Under the experimental conditions employed the administered progesterone is rapidly metabolized and excreted by male cats. A most striking feature of the results is that whereas up to 67·1% of the dose appeared as biliary metabolites, in no animal did urinary radioactivity exceed 1%. Rats and mice excrete an oral, intramuscular or intraperitoneal dose of [21-$^{14}$C]progesterone mainly in the bile, but 22–34% appears in the urine (Grady *et al.* 1952; Shen *et al.* 1954). This difference between cats and rodents may be related to the fact that the former were anaesthetized during the whole period of the experiment whereas the latter were not. Also the steroid was administered intravenously to the cats but by other routes to the rodents. However, Sandberg & Slaunwhite (1958) and Wiest *et al.* (1958) administered [4-$^{14}$C]progesterone intravenously to human subjects and found a considerable, and usually larger, proportion of radioactive metabolites in the urine. Therefore it would seem that excretion of progesterone metabolites by the cat differs from that by rodents and human subjects. But other factors may be involved in this apparent species difference. The cats were anaesthetized with chloralose and urethane, both of which have been reported to act as choleretics, although their use is recommended for studies on bile production (Sobotka, 1937). Also, it is well established that enterohepatic circulation of steroid-hormone metabolites occurs under normal conditions, but in the present investigation such circulation could not occur since no bile reached the gastrointestinal tract. Grady *et al.* (1952) and Shen *et al.* (1954), however, found that in rodents urinary excretion of metabolites of [21-$^{14}$C]progesterone appeared to be independent of the enterohepatic circulation, and the same conclusion for human subjects was drawn by Sandberg & Slaunwhite (1958). It might also be supposed that the surgical procedures and anaesthetics used on the cats might have had some adverse effect on renal blood flow, thus preventing adequate urine formation and excretion of metabolites. But a good degree of renal function seems to have been maintained in the cats (Table 2), since urine volumes and total urinary solids compare favourably with those reported for normal, non-anaesthetized cats (Prentiss, Wolf & Eddy, 1959; Wolf, Prentiss, Douglas & Swett, 1959).

### *Nature of the conjugates*

The conjugates detected in the present study are those which it is generally accepted are formed during the metabolism of steroid hormones. Non-conjugated radioactivity was extractable before hydrolysis from both bile and urine, but a greater proportion of such excreted radioactivity was present in urine than in bile. Sandberg & Slaunwhite (1958) found that this also occurred in human subjects. It cannot be decided whether this radioactivity consists of unconjugated metabolites or of conjugates partially extractable from aqueous solution by ether.

Tables 1 and 2 show that appreciable amounts of neutral radioactivity became extractable by ether after incubation of bile and urine with limpet powder at pH 4·5. The limpet powder almost certainly contained sulphatases of various kinds (see Materials and Methods for reference), but the relative specificity of limpet steroid sulphatase and the fact that incubations were carried out in the presence of phosphate reduced the possibility of hydrolysis of steroid sulphate metabolites at this stage. Further evidence that glucuronides were present in bile and urine is provided by the results of the experiments shown in Table 3, which reveal almost complete inhibition by saccharate of release of radioactivity by limpet powder. Robinson & Williams (1958) found that cats did not excrete glucuronides in the urine after administration of a variety of non-steroidal substances known to be converted readily into such conjugates by other species of animals. Borrell (1958) investigated the urinary excretion of corticosteroids in cats, but observed no significant increase in Porter–Silber chromogens after incubation of the urine with $\beta$-glucuronidase. These findings could be reconciled with those of the present investigation by assuming that the cat excretes glucuronides almost exclusively in the bile. However, Dutton & Greig (1957) could not detect glucuronide formation in cat-liver slices under conditions in which the liver of other animals readily formed the conjugates of *o*-aminophenol and (−)-menthol. Therefore any firm conclusions about the identity of the glucuronides produced during the present investigation must await a fuller identification of the material hydrolysed by limpet powder.

It is generally recognized that sulphate esters of hydroxy steroids are readily hydrolysed by cold acid (Burstein & Lieberman, 1958), and it is probable that part at least of the radioactivity released by 2N-sulphuric acid was originally present as ester sulphates.

The nature of the metabolites released by hot-acid hydrolysis remains obscure. Any glucuronides and sulphates not hydrolysed in the previous steps would probably appear in this fraction, but the existence of conjugates other than these cannot be ruled out. Of particular interest is the presence in both bile and urine of appreciable amounts of radioactivity not extractable after all forms of hydrolysis. The urine contained a much higher proportion of such metabolites, in contrast with

human subjects in whom the bile contained a greater proportion of these metabolites (Sandberg & Slaunwhite, 1958). Those workers used chloroform as the extracting solvent and did not wash the extracts. In the present investigation it was noticed that at every stage of hydrolysis and extraction significant amounts of radioactivity were present in the alkali and aqueous washings of the ether extracts, hence these washings were always returned to the original aqueous solution before the next hydrolysis stage. This indicates that part at least of the non-extractable metabolites consists of phenolic or acidic substances, which may be non-hydrolysed conjugates or as yet unknown breakdown products of progesterone.

*Variation in excretion of conjugates with time*

Table 1 shows that when bile samples were collected for 1 or 2 hr. periods the proportions of the different conjugates in the samples varied with time. The most consistent variation was in glucuronide excretion, which showed progressive decrease in successive samples. A similar trend can be seen to occur in urine (Table 2), although insufficient samples were investigated to allow definite conclusions to be drawn. In some animals (nos. 1, 2 and 5) the fall in glucuronides was accompanied by an increase in cold-acid-hydrolysed metabolites, whereas in others (nos. 4 and 6) the proportion of these metabolites decreased in successive samples. No consistent change with time occurred in the proportion of hot-acid-hydrolysed radioactivity, but in four out of the five animals (nos. 2, 4, 5 and 6) there was a definite increase in the proportion of radioactivity present as non-extractable metabolites.

Many complex factors are involved in the secretion of substances into the bile. These include the concentration of the substances in the blood, the rate of uptake by the liver cells, the length of time they are bound by intracellular protein and the mechanism of secretion into the bile, whether by simple diffusion or by some active secretory mechanism. Sperber (1959) has discussed these factors at length, particularly with respect to the excretion of organic anions in the bile. It is not possible to say how the different proportions of conjugates detected in successive bile samples in the present investigation are related to the above factors. A knowledge of the rate of clearance of the various conjugates from the blood would have been of value, but it was felt that withdrawal of serial blood samples might have aggravated the effects of the experimental procedures by decreasing hepatic and renal blood flow. The splanchnic nerves of the animals had in fact been cut in order to minimize vasomotor effects of the operation and anaesthetic on splanchnic blood flow. Therefore, for instance, it cannot be decided whether the higher proportion of glucuronides appearing in early bile samples and the higher proportion of water-soluble radioactivity in the later samples was the result of differences in rate of uptake of progesterone or metabolites, or both, from the blood, the degree of binding with intracellular protein and the rate of metabolism, or in the rate of secretion by the liver cells. It is well established that competition for secretion into the bile occurs, particularly with organic anions (Sperber, 1959), and this competition may play some part in controlling the secretion of the progesterone metabolites.

Hsia, Dowben & Shaw (1960) showed that certain progesterone metabolites, as well as synthetic and natural progestational agents, can inhibit the conjugation with glucuronic acid of *o*-aminophenol, phenolphthalein and bilirubin *in vitro* by rat and guinea-pig liver. Thus the fall in proportion of glucuronide excreted may to some extent be due to an inhibitory effect of the administered progesterone and its metabolites on the conjugating mechanism in the liver of the experimental animals.

It is apparent therefore that, under the experimental conditions employed, the bile is the major route of excretion for progesterone metabolites in the cat. It would be of interest to determine whether other steroid-hormone metabolites are excreted in the bile to the same extent by this animal. In human subjects approximately 50 % of an intravenous dose of progesterone is excreted as urinary metabolites, whereas, under similar conditions, 80–90 % of a dose of a variety of other steroids, including corticosteroids, androgens and oestrogens, is excreted in the urine (see Sandberg & Slaunwhite, 1958, for reference). The experiments described confirm that, in man and other animals, progesterone is metabolized and excreted by a number of pathways.

## SUMMARY

1. [4-$^{14}$C]Progesterone has been administered intravenously to anaesthetized male cats as a single injection or as a 1 or 3 hr. infusion. Bile and urine were collected for periods of up to 6 hr. after the start of the injection, and the excretion of radioactive metabolites was determined.

2. The major part of the dose of progesterone (up to 67·1 %) was excreted in the bile, whereas in no cat did urinary excretion of metabolites exceed 1 %.

3. Bile and urine samples were hydrolysed by standard methods so that the following ether-soluble neutral fractions were obtained: non-conjugated (extractable before hydrolysis); glucuronide, extractable after incubation with limpet

powder; cold-acid hydrolysed; hot-acid hydrolysed. In addition there was a significant quantity of metabolites remaining water-soluble after all forms of hydrolysis.

4. When 1 or 2 hr. fractions of bile were analysed the proportion of radioactivity excreted as glucuronide always decreased in successive samples, whereas water-soluble metabolites generally increased. In some animals the proportion of cold-acid-hydrolysed metabolites increased and in others decreased. No consistent change was detectable in hot-acid-hydrolysed radioactivity. A similar trend was seen in successive urine samples.

5. The relationship of these results to similar studies of progesterone in man and other animals is discussed.

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# Purification and Properties of Muscle Phosphoglycerate Kinase

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The enzyme phosphoglycerate kinase catalyses the reversible transphosphorylation:

Phosphoglyceryl phosphate + ADP ⇌ 3-phosphoglyceric acid + ATP

This reaction is an integral part of the Embden–Meyerhof glycolytic scheme. The existence of the reaction was originally mentioned by Warburg & Christian (1939), and the first detailed investigations of the enzyme involved were made by Bücher (1947). The kinase has been partially purified from pea seeds by Bandurski & Axelrod (1953) and has been detected in blood by Blancher, Williams & Weiss (1955) and in liver by Ichihara & Greenberg (1957). Some statements about the behaviour of this enzyme upon fractionation of an aqueous extract of muscle have been made by Beisenherz *et al.* (1953), but, despite some erroneous statements in the literature, he has not described the crystallization of phosphoglycerate kinase. The present paper deals with the purification and properties of the enzyme from rabbit skeletal muscle. Some differences in properties of the enzymes from yeast and from muscle are also described. A preliminary report was presented earlier by Rao & Oesper (1959).

## MATERIALS AND METHODS

*Abbreviation.* $P_i$, Inorganic phosphate.

Unless otherwise stated, all reagents were obtained from Sigma Chemical Co., St Louis, Mo., or Schwarz Bio-Research Inc., Mount Vernon, New York. All optical

measurements were made with a Beckman model DU spectrophotometer. The barium salts of the various nucleotides and of phosphoglyceric acid were dissolved with HCl and converted into the sodium salts by treatment with a stoicheiometric amount of $Na_2SO_4$. The $BaSO_4$ was removed by centrifuging at 0–5° and the solution was neutralized to pH 7 with NaOH. However, for a study of the metal requirement and of nucleotide specificity, the solutions were neutralized with tris. All solutions were stored at 0–5°. $P_i$ was determined by the method of Lohmann & Jendrassik (1926). Glyceraldehyde 3-phosphate was made enzymically by the method of Meyerhof & Junowicz-Kocholaty (1943), and dihydroxyacetone phosphate according to Oesper & Meyerhof (1950). Phosphoglyceryl phosphate was prepared by the method of Negelein & Brömel (1939) with the modification of Oesper (1954). A neutralized solution was kept frozen at −20° and used as required. It was convenient to distribute the stock solution into several small tubes, rather than freeze and thaw the bulk of the solution repeatedly. The concentration was determined by the method of Negelein & Brömel (1939). An extinction coefficient of $6{\cdot}27 \times 10^6$ cm.²/mole for DPNH has been used in all calculations.

*Buffer systems.* Imidazole was a product of Eastman Kodak Co., Rochester, N.Y., U.S.A. Tris was a product of Sigma Chemical Co. Veronal was a product of Abbot Laboratories, Chicago, Ill.

*Inhibitors.* *p*-Chloromercuribenzoic acid, *p*-chloromercuriphenylsulphonic acid and iodosobenzoic acid were products of Sigma Chemical Co. Iodobenzoic acid was a product of Eastman Kodak Co. Phenyloxoarsine was a product of Delta Chemical Works and a gift of Dr J. S. Roth. Oxophenarsine hydrochloride was a product of Parke Davis Company, Detroit, Mich.; dichlorophenarsine hydrochloride, of Abbot Laboratories; *N*-ethylmaleimide, of Schwarz BioResearch Co.; and iodoacetamide, of Eastern Chemical Corp., Newark 2 N.J.

*Estimation of protein.* Protein was determined by the method of Robinson & Hogden (1940) after precipitation with trichloroacetic acid. Human serum albumin was used as a standard. In some cases, protein was also determined by the method of Warburg & Christian (1941), with corrections for nucleic acids as suggested by Kalckar (1947).

*Determination of enzyme activity.* The enzyme reactions were run at room temperature (23–27°). The enzymes were diluted in 0·2% human serum albumin just before use, because the highly purified yeast enzyme (Bücher, 1947) lost 70% of its activity in dilute buffer solutions over a period of 3–4 hr. at 0°. The purified enzyme from muscle was more stable. Enzymes from both sources were definitely more stable in 0·2% albumin solution.

*Back-reaction: assay method I.* The back-reaction was studied by the method of Bücher (1947) in an optical assay system that utilized the two reactions:

$$\text{Phosphoglycerate} + \text{ATP} \rightarrow \text{phosphoglyceryl phosphate} + \text{ADP} \quad (1)$$

$$\text{Phosphoglyceryl phosphate} + \text{DPNH} + \text{H}^+ \rightarrow \text{glyceraldehyde 3-phosphate} + \text{DPN}^+ + \text{P}_i \quad (2)$$

Sum:

$$\text{Phosphoglycerate} + \text{ATP} + \text{DPNH} + \text{H}^+ \rightarrow \text{glyceraldehyde 3-phosphate} + \text{P}_i + \text{DPN}^+ + \text{ADP} \quad (3)$$

Reaction (1) is catalysed by phosphoglycerate kinase and reaction (2) by glyceraldehyde 3-phosphate dehydrogenase.

The reaction mixture consisted of 50 $\mu$moles of imidazole or tris buffer, pH 7·0, 10 $\mu$moles of glutathione, 16 $\mu$moles of $Mg^{2+}$ ions, 13·25 $\mu$moles of phosphoglycerate, 4·65 $\mu$moles of ATP, 0·5 $\mu$mole of DPNH and an excess of glyceraldehyde 3-phosphate dehydrogenase (Krebs & Najjar, 1948) in a total volume of 2·8 ml. The kinase (0·2 ml.; diluted to give an extinction change of 0·12–0·15/min.) was added to start the reaction, which was followed by determining the extinction change at 340 m$\mu$ every 30 sec. for a period of 4 min. All reactions were run at two different concentrations of enzyme.

One unit of enzyme activity is defined as an extinction change of 0·10/min. under the above assay conditions.

Specific activity is defined as the number of units/mg. of protein. The change in extinction used to calculate the specific activity is either from $\frac{1}{2}$ to $1\frac{1}{2}$ min. or from 1 to 2 min. During this interval the rate is linear and proportional to enzyme concentration.

*Assay method II.* The method described is essentially that devised by Bandurski & Axelrod (1953).

A mixture was prepared, containing 50 $\mu$moles of imidazole buffer, pH 7·1, 12·5 $\mu$moles of phosphoglycerate, 4·5 $\mu$moles of ATP, 10 $\mu$moles of $MgCl_2$ and 750 $\mu$moles of hydroxylamine in 2 ml. of solution. The final pH was adjusted to 7·1, if necessary, by cautious addition of N-HCl, and the mixture equilibrated in centrifuge tubes in a water bath at 37° for 5 min. At appropriate time intervals, suitably diluted enzyme preparations were added so that the final volume was 2·2 ml. and the tubes were incubated for 30 min. at 37°. The blanks contained all components except the enzyme. At the end of this period, 3 ml. of $FeCl_3$–HCl–trichloroacetic acid (8·3 g. of $FeCl_3,6H_2O$, 20 g. of trichloroacetic acid and 42 ml. of conc. HCl in 500 ml. of water) was added, followed after 5 min. by 2·5 ml. of 95% ethanol. The mixture was stirred and centrifuged to remove denatured protein, and the absorption of the supernatant at 490 m$\mu$, which was due to the $Fe^{3+}$–phosphoglycerohydroxamic acid complex, was determined in a Beckman spectrophotometer. A large-scale incubation was also carried out, essentially by the same procedure, and the phosphoglycerohydroxamic acid identified by paper chromatography.

The hydroxylamine stock solution was made by the method of Beinert *et al.* (1953); it was not neutralized, but frozen in solid $CO_2$ and stored at −20°. The concentration of hydroxylamine was determined by titration with standard HCl.

Specific activity is defined as the extinction change/mg. of protein under the above assay conditions. The method is relatively insensitive, requires long incubation periods and is wasteful of material, but was used to determine the distribution of enzyme in various tissue extracts, and also to study the effect of inhibitors and metal ions on the enzyme. Assay method I is not suitable for crude extracts because the triose phosphate isomerase present will convert the glyceraldehyde phosphate into dihydroxyacetone phosphate, which reacts with DPNH in the presence of α-glycerophosphate dehydrogenase, which may also be present in the crude preparation. The change in extinction is, accordingly, quite misleading.

*Forward reaction.* The reaction in the forward direction can be studied by assay method I but is liable to inter-

ference by contaminating enzymes, especially triose phosphate isomerase and α-glycerophosphate dehydrogenase (Bücher, 1947) (see above). Consequently a separate assay method was devised.

*Assay method III.* The reaction mixture contained 50 μmoles of imidazole buffer, pH 7·0, 4·68 μmoles of ADP, 8 μmoles of $Mg^{2+}$ ions, 0·1 or 0·2 ml. of a suitably diluted enzyme preparation in a volume of 1·8 ml. The reaction was started by adding 2 μmoles of phosphoglyceryl phosphate. The final volume was 2 ml. At the end of 5 min. at room temperature (23–27°) 0·3 ml. of 30% (w/v) trichloroacetic acid was added, the tubes were well shaken and centrifuged, and the inorganic phosphate was determined. The acyl phosphate of phosphoglyceryl phosphate is hydrolysed by the acid molybdate in the phosphate reagent, whereas the ATP into which it is converted is not, so that the extent of reaction is immediately given by this assay.

*Determination of Michaelis constants lying below* $10^{-4}$ *moles/l.* The Michaelis constant for phosphoglyceryl phosphate is so small that it is impossible to measure it directly; when the concentration of substrate is small enough to affect the reaction rate, the amount of reaction is too small to measure by ordinary means (spectrophotometry or phosphate determinations).

We have devised an isotopic method for measuring the reaction rate at very low substrate concentrations. In this procedure, the enzyme is incubated with labelled substrate, the reaction stopped, a known amount of unlabelled product added, and the specific activity of the purified product is determined; the activity of the product is obviously proportional to the extent of the reaction.

In our particular case, phosphoglyceryl phosphate containing $^{32}P$ in the 1-position was incubated with enzyme and excess of ADP for 30 sec. at 25° in 5 ml. of 0·05 M-tris buffer, pH 7. The reaction was stopped with 1 ml. of 30% trichloroacetic acid, ATP and $P_i$ were added as carriers, the mixture was heated at 60° for 30 min. to decompose the remaining phosphoglyceryl phosphate, and centrifuged to remove the coagulated protein. The pH was adjusted to 1·7 and the ATP precipitated at 0° with 10 vol. of acetone. The ATP was redissolved in water and reprecipitated twice more, additional carrier $P_i$ being added before each reprecipitation. The carrier $P_i$ dilutes out the $[^{32}P]P_i$ derived from the active phosphoglyceryl phosphate, so that the ATP finally isolated is essentially free of the activity of the phosphoglyceryl phosphate remaining at the end of the reaction. Details of the separation of ATP from $P_i$ are described by Oesper (1954).

## RESULTS

### *Activity of the enzyme in different tissues*

Table 1 shows the activity of phosphoglycerate kinase in various tissues of the rabbit, studied by assay method II.

### *Preparation of phosphoglycerate kinase*

Rabbit skeletal muscle was used as the source of phosphoglycerate kinase because of its high initial specific activity and easy availability. Initial attempts to purify the enzyme by ammonium sulphate fractionation were unsuccessful, as Beisenherz *et al.* (1953) had also found. Therefore, attempts were made to develop a method for the isolation of the enzyme by fractionation with organic solvents.

All procedures were carried out at the temperatures noted below. Contact of the solutions and various fractions with metal was avoided throughout the fractionation. Measurements of pH were made at or near 20°, with a Beckman pH meter. Whenever a protein-precipitating agent was used, the mixture was stirred vigorously but without foaming, and kept for at least 5 min. before centrifuging. The concentration of acetone is expressed in terms of volume per cent, assuming that the volumes are additive. The temperature during the acetone fractionations was held at −5°. A typical protocol is as follows

*Extraction of tissue.* A rabbit was anaesthetized by intravenous injection of a 5% solution of sodium pentobarbital. The carotid artery was cut and the animal bled. The leg and back muscles were dissected in a cold room at 2–4° and passed twice through a chilled meat grinder; 430 g. of muscle was obtained. It was stirred with twice its volume (860 ml.) of water and kept at 2–5° for 3 hr. with occasional stirring. The suspension was centrifuged at 3500*g* for 25 min. and the clear supernatant decanted through glass wool.

*Acetone fractionation;* '40% *acetone' fraction.* To 800 ml. of muscle extract at approximately 0°, 534 ml. of chilled acetone (below −20°) was added over a period of 10–15 min. at −5°. The precipitate had essentially no activity and over 40% of the total protein was removed in this step.

'45% *acetone' fraction.* Chilled acetone (120 ml.; about −10°) was added to the supernatant from the previous step over about 5 min. and the mixture centrifuged at 1020*g* for 25 min. at −5°. The precipitate had about 25% of the original activity but the specific activity was low. The clear supernatant was used for the next step.

'50% *acetone' fraction.* Chilled acetone (146 ml.; about −10°) was added to the supernatant from

Table 1. *Activity of phosphoglycerate kinase in various tissues*

Rabbit liver, kidney, brain and muscle were dissected out immediately after killing the animal with sodium pentobarbital. The tissues were weighed and homogenized with 2½ vol. of water at 0–5° in a glass homogenizer. The homogenate was centrifuged at 3500*g* for 15 min. and the clear supernatant used for the determination of the activity by assay method II.

| Tissue | Activity/g. of tissue | Activity/g. of protein |
|---|---|---|
| Muscle | 27·3 | 624 |
| Brain | 14·0 | 320 |
| Kidney | 12·9 | 114 |
| Liver | 12·8 | 81 |

the previous step over about 5 min. and the mixture centrifuged at 1020*g* for 30 min. at −5°. The supernatant was rejected and the precipitate was dissolved in 30 ml. of ice-cold water, frozen and freeze-dried to remove acetone. The freeze-dried material could be stored at −20° for a day or two. It was dissolved in ice-cold water and centrifuged at 3500*g* to remove any insoluble material. The clear solution was used for the next step.

*Heating in the presence of ammonium sulphate.* The volume of the solution was adjusted to give a protein concentration of 10 mg./ml. Solid ammonium sulphate was added to give a final concentration of 10 %. The pH was 6·0–7·0. The solution was heated to 50–52° for 5 min. in a water bath, cooled to 10°, centrifuged to remove denatured protein, and decanted through glass wool. This fraction could be kept frozen at −20° for a month without loss of activity.

*Adsorption on alumina Cγ.* The above fraction was dialysed to remove ammonium sulphate and the pH adjusted to 8·0 with 0·5M-tris. Alumina Cγ gel (Willstätter & Kraut, 1923) was added (protein: gel ratio, 5–6:1). The mixture was stirred in the cold room at 2–5° for 20 min. with a magnetic stirrer and centrifuged at 3000*g*. The packed gel was eluted successively with 0·5, 1, 2, 4, 5, 7·5 and 10 % ammonium sulphate solutions and each of the fractions was assayed separately. In a typical experiment 66 ml. of a solution containing 225 mg. of protein was stirred with 1300 mg. of alumina Cγ gel as described above. Twenty per cent of the activity was recovered in the supernatant and the purification was about twofold. Ammonium sulphate solution (25 ml.) was used for each elution. The first fraction contained 6 % of the activity and the purification was threefold. Succeeding fractions had lower specific activity.

The conditions for the gel-adsorption step are rather critical and the results variable. The gel-adsorption procedure eliminated the trace of α-glycerophosphate-dehydrogenase activity present in the earlier fraction.

A summary of results of a typical fractionation procedure is given in Table 2.

*Properties of the purified phosphoglycerate kinase*

Most of the experiments as well as the properties recorded here apply to the preparations that were not subjected to purification by alumina Cγ-gel procedures but were carried through the heating step.

*Tests for the presence of other enzymes in the phosphoglycerate-kinase preparation.* The enzyme fractions after heat treatment were free of aldolase, triose phosphate dehydrogenase, DPNH oxidase, adenosine triphosphatase, myokinase and acyl phosphatase (Harary, 1957) when tested at concentrations 100 times greater than that used in the standard-activity determination (method I). Triose phosphate isomerase and a trace of α-glycerophosphate dehydrogenase were detected in all enzyme preparations. The latter could be removed by the treatment with alumina Cγ.

*Stability.* The enzyme could be repeatedly frozen and thawed over short intervals without loss of activity. About 50 % of the activity was lost in a period of 3 months with repeated freezing and thawing. The enzyme was stable in the frozen state in the presence of ammonium sulphate.

*Activation by metal ions.* The effect of cations on the activity of phosphoglycerate kinase was tested by assay methods I and III. Both muscle and yeast kinases required $Mg^{2+}$ or $Mn^{2+}$ ions (approximately 2 mM) for full activity. Higher concentrations of the metal ions were slightly inhibitory. Even after extensive dialysis, both kinases showed a small residual activity, which could be completely eliminated by adding EDTA. The inhibitory effect of EDTA could be abolished by adding $Mg^{2+}$ or $Mn^{2+}$ ions. There was no activation of either muscle or yeast kinase by $K^+$, $Li^+$, $Zn^{2+}$, $Co^{2+}$, $Ni^{2+}$ or $Al^{3+}$ ions.

*Nucleotide specificity.* The nucleotide specificity of the muscle phosphoglycerate kinase was investigated by assay methods I and III. The enzyme is specific for ATP and ADP in the back and forward reactions respectively. None of the other adenine nucleotides (adenosine 2′-, 3′- or 5′-monophosphate) were effective in replacing ATP or ADP. Uridine triphosphate, inosinic acid and creatine phosphate were also ineffective. Inosine triphosphate, however, did have some activity (up to 40 % of that found with ATP). ADP was not active in the back-reaction.

*Effect of inhibitors on phosphoglycerate-kinase activity.* The effect of inhibitors on the muscle and yeast kinases was studied in the back and forward

Table 2. *Purification of phosphoglycerate kinase from muscle*

Assay method I was used to follow the degree of purification.

| Fraction | Total protein (g.) | Specific activity | Purification | Total units | Recovery (%) |
|---|---|---|---|---|---|
| Muscle extract | 13·28 | 24 | 0 | 319 900 | 100 |
| '50 % acetone' fraction | 0·640 | 135 | 5·6 | 86 400 | 27 |
| Heat treatment at 50–52° | 0·484 | 164 | 7·0 | 79 380 | 24 |

reactions, by assay methods II and III. The muscle kinase is highly sensitive to *p*-chloromercuribenzoate and *p*-chloromercuriphenylsulphonic acid; 100% inhibition was obtained at concentrations of 0·26 and 0·43 mM respectively. The yeast enzyme, however, was not inhibited even by 10 mM-*p*-chloromercuribenzoate. The activities of both the muscle and yeast enzymes were unaffected by iodoacetamide, *o*-iodobenzoate, iodosobenzoate or *N*-ethylmaleimide at concentrations of 4·36 mM. Oxophenarsine hydrochloride, dichlorophenarsine hydrochloride and phenyloxoarsine had no effect at concentrations of 1 mM.

The inhibition due to mercurial sulphydryl reagents could be reversed by addition of GSH or cysteine. A slight activating effect of EDTA was also noticed in some instances in the presence of excess of $Mg^{2+}$ ions. This is presumably due to the removal of heavy-metal contaminants in the assay system or enzyme.

*Determination of Michaelis constants for phosphoglyceric acids, adenosine tri- and di-phosphate, and $Mg^{2+}$ and $Mn^{2+}$ ions.* The Michaelis constants for phosphoglycerate, ATP, $Mg^{2+}$ and $Mn^{2+}$ ions were determined in the back-reaction, by assay method I. The Michaelis constant for ADP was determined in the forward-reaction, by the assay method described by Bücher (1947) for the yeast enzyme, and method III for the muscle enzyme.

As we have already observed, the Michaelis constant for phosphoglyceryl phosphate is so small that it is impossible to measure it in the usual way; Bücher (1947) overcame this difficulty by measuring the competition between phosphoglyceryl phosphate and phosphoglycerate or ATP, and computing what the enzyme–substrate dissociation

Table 3. *Isotopic determination of the Michaelis constant of yeast phosphoglycerate kinase for phosphoglyceryl phosphate*

Phosphoglyceryl phosphate (activity, 4 820 000 counts/min./μmole) was incubated at the concentrations given below, in the presence of 1·5 mM-ADP and 0·033 μg. of enzyme. Total volume equalled 5 ml.; 44·5 μmoles of ATP and 49 μmoles of $P_i$ were added as carriers, with an additional 147 μmoles of $P_i$ being added before each of the two reprecipitations of ATP.

| Phosphoglyceryl phosphate concn. (μmole/ml.) | Total counts/min. recovered* | ATP recovered (μmoles) | Counts/min./μmole of ATP | ATP formed/ml. of reaction mixture (μmole)† |
|---|---|---|---|---|
| 0·00516 | 4643 | 21·7 | 214 | 0·000395 |
| 0·00258 | 3202 | 20·4 | 157 | 0·000291 |
| 0·00134 | 2517 | 21·7 | 116 | 0·000215 |

* Corrected for background and blank.

† Counts/min./μmole × (44·5/4 820 000) × ($\frac{1}{5}$).

Table 4. *Isotopic determination of the Michaelis constant of muscle phosphoglycerate kinase for phosphoglyceryl phosphate*

Experiment similar to that described in Table 3. Activity of phosphoglyceryl phosphate was 1 570 000 counts/min./μmole; volume, 4·0 ml.; ADP, 2 mM; 0·02 μg. of enzyme. Carrier ATP (51·4 μmoles) was added.

| Phosphoglyceryl phosphate concn. (μmole/ml.) | Total counts/min. recovered* | ATP recovered (μmoles) | Counts/min./μmole of ATP | ATP formed/ml. of reaction mixture (μmole)† |
|---|---|---|---|---|
| 0·00495 | 1040 | 24·6 | 42·3 | 0·000346 |
| 0·00248 | 570 | 27·3 | 20·9 | 0·000171 |
| 0·00124 | 390 | 20·4 | 19·1 | 0·000156 |

* Corrected for background and blank.

† Counts/min./μmole × (51·4/1 570 000) × ($\frac{1}{4}$).

Table 5. *Michaelis constants for substrates and activators*

$K_m$ values are given in moles/l.

| Enzyme source | pH | Phospho-glycerate | ATP | Phosphoglyceryl phosphate | ADP | $Mg^{2+}$ | $Mn^{2+}$ |
|---|---|---|---|---|---|---|---|
| Yeast | 6·0 | $8·5 \times 10^{-4}$ | — | — | — | — | — |
| Yeast | 7·0 | $13·0 \times 10^{-4}$ | $3·2 \times 10^{-4}$ | $2·0 \times 10^{-6}$ | $4·0 \times 10^{-4}$ | $2·8 \times 10^{-4}$ | $5·9 \times 10^{-4}$ |
| Yeast | 6·9* | $2·0 \times 10^{-4}$ | $1·1 \times 10^{-4}$ | $1·8 \times 10^{-6}$ | $2·0 \times 10^{-4}$ | $2·5 \times 10^{-4}$ | — |
| Yeast | 8·0 | $10·7 \times 10^{-4}$ | $3·3 \times 10^{-4}$ | — | — | — | — |
| Muscle | 6·0 | $13·6 \times 10^{-4}$ | — | — | — | — | — |
| Muscle | 7·0 | $12·2 \times 10^{-4}$ | $4·8 \times 10^{-4}$ | $2·2 \times 10^{-6}$ | $3·5 \times 10^{-4}$ | $2·7 \times 10^{-4}$ | $3·0 \times 10^{-4}$ |
| Muscle | 8·0 | $10·7 \times 10^{-4}$ | $4·8 \times 10^{-4}$ | — | — | — | — |
| Pea seeds | 7·3† | $7·6 \times 10^{-3}$ | $4·1 \times 10^{-3}$ | — | — | — | — |

* Bücher (1947).

† Bandurski & Axelrod (1953).

constant for phosphoglyceryl phosphate must have been. We have used the isotopic method described above. Typical protocols are given in Tables 3 and 4.

The $K_m$ values for the various substrates and activators are given in Table 5, the results having been obtained from graphs drawn according to Dixon (1953).

## DISCUSSION

The present work has shown that a protein possessing phosphoglycerate-kinase activity can be precipitated from an aqueous extract of rabbit skeletal muscle by fractionation with acetone. About 30% of the total activity was recovered in the 50% acetone fraction, an approximately six-fold purification being obtained in this single step. In general, ammonium sulphate fractionation has proved unsatisfactory for purifying transphosphorylating enzymes. Similar observations were made by Askonas (1951), who adopted organic solvents such as acetone, propanol etc. for the purification. Kuby, Noda & Lardy (1954) and Noda & Kuby (1957) have used acetone fractionation in the presence of bivalent metal ions such as $Mg^{2+}$, $Mn^{2+}$ or $Zn^{2+}$ (for which the enzyme protein has a high affinity) and have succeeded in obtaining crystalline preparations of creatine phosphokinase and myokinase.

The entire fractionation procedure was highly reproducible up to the alumina-gel stage, and an overall purification of 12- to 15-fold was repeatedly obtained after the gel treatment. Further attempts to purify the enzyme with organic solvents in the presence of $Mg^{2+}$, $Mn^{2+}$ or $Zn^{2+}$ ions, by isoelectric precipitation, selective denaturation of inactive protein with chloroform or by subsequent ammonium sulphate fractionations, were not encouraging, Failure to achieve further purification by these methods raised the question whether the phosphoglycerate kinase might be a particulate enzyme like brain hexokinase (Crane & Sols, 1953). However, repeated centrifugings for various lengths of time at 100 000*g* did not change the specific activity of the enzyme. The purified kinase from rabbit skeletal muscle was contaminated with triose phosphate-isomerase activity and was not electrophoretically homogeneous.

There is an absolute requirement for a bivalent metal ion such as $Mg^{2+}$ or $Mn^{2+}$. For the muscle enzyme $Mg^{2+}$ ions are more effective than $Mn^{2+}$ ions at high concentrations, whereas at low concentrations $Mn^{2+}$ ions are more effective. Lardy & Ziegler (1945) observed the effect of $K^+$ ions on pyruvic phosphokinase in dialysed muscle extracts but thought that phosphoglycerate kinase was probably not activated by $K^+$. These findings have now been confirmed with purified phosphoglycerate kinase from muscle.

The inhibition of muscle phosphoglycerate kinase by –SH group-binding reagents like *p*-chloromercuribenzoate, *p*-chloromercuriphenylsulphonic acid, and heavy-metal ions like $Cu^{2+}$ ions indicates the need for –SH groups for the activity of the enzyme. The lack of inhibition by organic arsenicals or iodoacetamide is not surprising, since there are instances of –SH group enzymes that are highly sensitive to *p*-chloromercuribenzoate but not to organic arsenicals or iodoacetamide (Rose, Grunberg-Manago, Korey & Ochoa, 1954). Since both the yeast and muscle enzymes catalyse the same reaction, but only the latter appears to need –SH groups for activity, interesting possibilities regarding the mechanism of action of the enzymes arise. Dixon (1949) has proposed a mechanism for transphosphorylation, in which –SH groups combine to form –SH–magnesium–phosphate complexes. The more recent proposals of Koshland (1954) or Boyer & Harrison (1954) do not appear to assign any role to the –SH group in transphosphorylation reactions.

Some aspects of the substrate and nucleotide specificity of the phosphoglycerate-kinase reaction have been investigated, and, on the basis of the present work and the findings of Bandurski & Axelrod (1953), one may state that the enzymes from three different sources (plants, yeast and muscle) are absolutely specific for phosphoglycerate, ATP, ADP and phosphoglyceryl phosphate. The slight activity noticed with inosine triphosphate may be due to the presence of ADP or ATP in the sample. If it is due to ADP this would imply the presence of a nucleoside diphosphokinase in the phosphoglycerate kinase which would catalyse the reaction:

$$\text{Inosine triphosphate} + \text{ADP} \rightarrow \text{inosine diphosphate} + \text{ATP}.$$

The Michaelis constants determined in the present study for all the substrates and activators of both yeast and muscle enzymes are comparable with the values reported by Bücher (1947). The Michaelis constant for phosphoglyceryl phosphate determined here by an isotopic method agrees closely with the value found by Bücher from studies of competitive inhibition. It is also consistent with direct measurements of the binding of the enzyme to the substrate (Oesper, 1961).

## SUMMARY

1. A partial purification of the enzyme, phosphoglycerate kinase, from an aqueous extract of rabbit skeletal muscle has been achieved. The purified enzyme was not homogeneous electrophoretically and contained two components. It was free of most of the enzymes of the anaerobic

glycolytic pathway but contained triose phosphate isomerase.

2. A new assay method for studying the phosphoglycerate-kinase reaction in the forward direction was developed. The method has the advantage that it can be used with partially purified enzyme preparations containing triose phosphate isomerase and α-glycerophosphate dehydrogenase. The assay method also facilitated an independent study of inhibitors and metal ions in the enzyme reactions.

3. Phosphoglycerate kinase is activated by $Mg^{2+}$ and $Mn^{2+}$ ions, and by no other metal ions tested. It reacts specifically with adenosine and di- and tri-phosphate, but with no other nucleotides.

4. The study of the effect of various thiol-binding agents on the muscle and yeast phosphoglycerate kinases revealed that the muscle phosphoglycerate kinase is a sulphydryl enzyme whereas the yeast enzyme is not.

5. The Michaelis constants for the various substrates and activators obtained with the muscle enzyme are similar to the values obtained with the yeast enzyme. These values are also in good agreement with the data originally reported by Bücher (1947). The Michaelis constants of the muscle and yeast enzymes for phosphoglyceryl phosphate have been determined independently by an isotopic method.

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# Digestion and Absorption of Disaccharides in Man

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Disaccharides and starch are well known to constitute the major part of the carbohydrates present in our diet. The general conception of the digestion of disaccharides, as concluded from current text-books of biochemistry, is that disaccharide hydrolases (glycosidases) are secreted with the 'succus entericus' into the intestinal lumen, where the hydrolysis of disaccharides is believed to occur.

During a recent study on intestinal digestion and absorption in man (Borgström, Dahlqvist, Lundh & Sjövall, 1957), it was observed, however, that the glycosidase activity of the intestinal contents during digestion was too low to account for any

considerable digestion of disaccharides. In spite of this, a disaccharide, lactose, was rapidly absorbed when given in the test meal. These findings suggested that the intestinal glycosidases were present in the cells of the intestinal mucosa, and were in good accordance with the results obtained by some previous authors (Cajori, 1933; Ammon & Henning, 1956).

We have now performed a series of experiments in which sucrose, maltose, lactose or, in a few cases, soluble starch have been given in a test meal to men, together with fat, protein and an unabsorbable reference substance for calculating the dilution of the test meal in the intestine. The hydrolysis and the absorption of the carbohydrates, and the carbohydrase activities of the intestinal contents, have been assayed in samples obtained from different levels of the intestine through a plastic tube.

## MATERIALS AND METHODS

Twenty-one experiments were performed on 17 male students, 19–29 years old, weighing 58–78 kg. and having a height of 170–188 cm.

### *Intubation technique*

The technique for intubation was essentially that of Blankenhorn, Hirsch & Ahrens (1955), as was used by Borgström *et al.* (1957). The tube was marked so that the distance from the nose to the place in the intestine from which the sample was obtained could be measured. The anatomical localization corresponding to this distance has been carefully investigated by Blankenhorn *et al.* (1955). In the present paper the distance from the nose to the pylorus is assumed to be 60–65 cm.; to the ligament of Treitz (which marks the limit between the duodenum and the jejunum) 85–90 cm.; to the ileocoecal valve 300 cm. In some experiments the balloon containing mercury was replaced by a small steel cylinder, 5 mm. × 14 mm., with hemispherical ends. Intubation was performed in the afternoon of the day before the experiment. In a few experiments the tube was left for 2 days, in order to reach the lower levels of the small intestine. After the experiment the tube was removed by pulling it out through the nose.

Before the test meal was given, the subjects fasted for 12 hr., but were allowed water.

### *Composition of the test meal*

The test meal was of liquid, containing carbohydrate (sucrose, maltose, lactose or soluble starch), fat (either lard or a vegetable oil containing chiefly unsaturated triglycerides), protein (dried egg white), polyethylene glycol (mol.wt. 4000) and egg yolk. The composition by weight of the meal given to each person is: dried egg white, 21·1 g. (contains 18·8 g. of protein), carbohydrate (expressed as the amount of monosaccharides which would be obtained on complete hydrolysis), 56·3 g., fat, 22·3 g., one egg yolk (weighing about 18 g., and containing 5 g. of fat and 3 g. of protein), polyethylene glycol, 4·0 g. and tap water, 295 g. The meal thus had a total weight of about 400 g. and contained 550 kcal.

The carbohydrates used were of analytical-grade purity; sucrose and lactose monohydrate were from Baker Co. (U.S.A.), maltose monohydrate from Pfanstiehl Chemical Co. (U.S.A.) and soluble starch ('amylum soluble nach Zulkowsky') was from Merck A.-G. (Germany). Polyethylene glycol was obtained commercially.

For preparation of the formula the carbohydrate, protein and polyethylene glycol were dissolved individually in water. The solutions then were mixed with the egg yolk in an Ultra-Turrax homogenizer, the fat being slowly added to the solution during homogenization to obtain a smooth emulsion.

The egg yolk was used to obtain a more stable emulsion. Polyethylene glycol was used as a reference substance for calculating the degree of dilution of the test meal in the samples obtained from the intestine, and thus allowing calculation of the degree of absorption of the carbohydrate. Polyethylene glycol passes through the intestinal canal without being absorbed (Borgström *et al.* 1957); it was determined turbidimetrically (Hydén, 1955).

The weight of each carbohydrate in the present paper is expressed as the weight of the monosaccharides which would be obtained on complete hydrolysis, i.e. 1·00 g. of carbohydrate means 0·95 g. of anhydrous disaccharide or 0·90 g. of starch.

### *Sampling*

After the test meal had been ingested, samples were collected for the next 3–4 hr. by siphoning into flasks surrounded by crushed ice. Samples were collected during periods of 30 or 60 min.

With the low glycosidase activity present in the intestinal contents this precaution was sufficient to prevent additional hydrolysis of disaccharides, but possibly not of starch (see below). Analysis for enzymic activity and carbohydrate contents was, generally, performed during the same day as the samples had been collected. Otherwise the samples were stored at −16°, at which they are stable for at least some weeks.

### *Assay of enzymic activities*

Invertase and lactase activities were assayed as described by Dahlqvist (1960), and maltase activity as described by Dahlqvist (1961*a*). One unit of glycosidase activity causes 5% hydrolysis (i.e. the formation of 1 mg. of hexose) in 2 ml. of reaction mixture during 60 min. at 37° at optimum pH in 28 mM substrate. The intestinal contents, usually diluted 1:5 or 1:10, were used for the glycosidase-activity determinations without any previous purification. Since the sample thus contained some disaccharide, only the glycosidase activity corresponding to the disaccharide administered was assayed in each case.

Amylase activity was determined as described by Dahlqvist (1961*b*). One unit of amylase activity causes an increase in reducing power corresponding to 1 mg. of maltose monohydrate in 60 min. at 37°, and is thus comparable with the unit for glycosidase activity. This is not the unit for amylase activity used by Borgström *et al.* (1957) with incubation for 3 min. at 25°.

### *Assay of carbohydrate*

Assay of carbohydrate was performed by different methods depending on the kind of carbohydrate present in the test meal. In all cases the amount of free hexose and

disaccharide were assayed *per se*, and in the experiments with starch the average degree of polymerization of the oligosaccharides present was determined in each sample.

*Protein precipitation.* To 0·5 ml. of the sample was added 1·5 ml. of water, 1·0 ml. of 5% (w/v) $ZnSO_4,7H_2O$ and 1·0 ml. of 0·3N-$Ba(OH)_2$ (Somogyi, 1945). After mixing and centrifuging, the protein-free supernatant was used for the determination of hexoses and disaccharides present. It could not, however, be used for the determination of starch, a major part of which was precipitated by these reagents. The protein-free supernatant was assumed to contain 0·125 ml. of the original sample/ml.

*Assay of sucrose and its hydrolysis products.* A suitable amount of the protein-free supernatant (0·5–1·0 ml.) was diluted with water to 2·0 ml. in a test tube. After the addition of 2·0 ml. of Sumner's (1924) 3:5-dinitrosalicylate reagent (most readily prepared as described by Hostettler, Borel & Deuel, 1951) the tube was immersed in a boiling-water bath for 10 min. and then chilled for 2 min. in running tap water. Then 20 ml. of water was added, the contents of the tube were mixed and the extinction at 530 mμ was measured in a spectrophotometer against a blank without sugar, with 1 cm. cuvettes. The amount of glucose and fructose present was calculated from a calibration curve obtained with known amounts (0·2–3·0 mg.) of glucose. Glucose and fructose have the same extinction coefficient with this reagent, but sucrose does not produce any colour. The amount of sugar measured by this method is thus the amount of free glucose and fructose present in the sample. The colour is stable for at least 10 hr.

For measuring the amount of sucrose present, 0·1–0·5 ml. of the protein-free supernatant was diluted with water to 1·0 ml., 1·0 ml. of buffered yeast invertase solution (10 mg. of invertase, analytical, Nutritional Biochemicals Corp., U.S.A., dissolved in 50 ml. of 0·1M-sodium acetate adjusted to pH 4·5 with 0·1M-acetic acid) was added and the tube immersed in a water bath at 37° for 1 hr. (The invertase activity was sufficient to hydrolyse 3 mg. of sucrose in less than 10 min.) Then 2·0 ml. of the 3:5-dinitrosalicylate reagent was added and the amount of reducing sugar present measured as described above. The amount of sucrose present in the sample was calculated from a standard series, prepared from glucose, after subtraction of the colour produced by the amount of free sugar originally present.

*Assay of maltose and its hydrolysis product.* The amount of free glucose in the presence of maltose was measured by the tris–glucose oxidase reagent described by Dahlqvist (1961*a*). A suitable amount of the protein-free supernatant (0·1–0·2 ml.), containing less than 2 mg. of maltose determined as described below, was diluted with water to 0·5 ml. After the addition of 3·0 ml. of the tris–glucose oxidase reagent (Dahlqvist, 1961*a*) the tube was immersed in a water bath at 37° for 1 hr. for development of the colour. The colour produced then was measured in a spectrophotometer at 420 mμ, and the amount of glucose present was calculated from a calibration curve, obtained with known amounts (0·01–0·05 mg.) of glucose.

To measure maltose the reducing power of 0·1–0·5 ml. of protein-free supernatant was determined with the 3:5-dinitrosalicylate reagent. A correction for the free glucose present was made and the amount of maltose calculated from a standard curve, obtained with 0·2–4·0 mg. of maltose.

*Assay of lactose and its hydrolysis products.* For assay of glucose and galactose in the presence of lactose, 0·1–0·2 ml. of the protein-free supernatant (containing less than 2 mg. of lactose as determined below) was diluted with water to 2·0 ml. in a Folin sugar tube, and 2·0 ml. of acid copper reagent (Tauber & Kleiner, 1932) was added. The tube was heated in a boiling-water bath for 10 min., and then chilled with running tap water for 2 min. Thereafter 2·0 ml. of the arsenomolybdate reagent of Nelson (1944) was added, the contents of the tube were mixed, diluted with water to the 25 ml. mark and mixed again. After the mixture had been standing at room temperature for 15 min. for the colour to be fully developed, reading was made in a spectrophotometer at 660 mμ. Glucose and galactose gave similar standard curves with this method. The standard curve was usually prepared from an equimolar mixture of glucose and galactose (0·1–0·5 mg.). Lactose in amounts less than 2 mg. did not produce any colour.

To determine lactose the reducing power of 0·1–0·2 ml. of protein-free supernatant was determined with the 3:5-dinitrosalicylate reagent. After correction for the colour produced by the glucose and galactose present (glucose and galactose have the same extinction coefficient with this method) the amount of lactose was calculated from a standard curve prepared with known amounts (0·2–4·0 mg.) of lactose.

*Assay of starch and its hydrolysis products.* These carbohydrates were determined with anthrone by a slight modification of the method described by Scott & Melvin (1953); 2·0 ml. of the sample to be assayed, diluted 1:1000 or more, was transferred to a test tube, which was chilled with tap water. With the tube still in the tap-water bath, 4·0 ml. of the anthrone reagent (100 mg. of anthrone freshly dissolved in 50 ml. of conc. sulphuric acid) was allowed to flow along the wall of the tube to its bottom, and then the contents of the tube were mixed slowly to avoid excessive heating. The tube was covered with an inverted 5 ml. beaker, immersed in a boiling-water bath for 7·5 min. and chilled with tap water for 15 min. The intensity of the colour produced was measured in a spectrophotometer at 625 mμ, with 1 cm. cuvettes. The amount of carbohydrate present was calculated from a standard series prepared from glucose (0·05–0·20 mg.).

Since protein-precipitation reagents also precipitated the major part of starch present in a solution, the anthrone determination had to be performed without protein precipitation. The sensitivity of the method, however, allowed so great dilution of the sample that the proteins present did not interfere. The solutions used for spectrophotometric readings were always perfectly clear. The secretions present in the intestine contained only negligible amounts of anthrone-positive material, that in the intestinal contents from a fasting subject corresponding to less than 1 mg. of glucose/ml. Determination of total carbohydrate with the anthrone reagent in a sample of intestinal contents containing sucrose gave a value in good accordance with that obtained by the method for assay of sucrose described above. Polyethylene glycol does not react with the anthrone reagent.

For determination of the amount of free glucose the tris–glucose oxidase reagent was used on deproteinized samples as described above.

For determination of the average degree of polymerization of the carbohydrate present in the experiments when

the test meal contained soluble starch, the reducing power was measured with the 3:5-dinitrosalicylate reagent. This was performed as described above, but 0·1–0·2 ml. of the sample was used without previous protein precipitation. This did not interfere with the production of the colour, although the solution in most cases became turbid. Before measurement of the intensity of the colour the solution was therefore shaken with 0·1–0·2 g. of Celite and then filtered. The filtrate was perfectly clear. In control experiments with pure sugar solutions, no colour was adsorbed on the Celite.

After correction of the colour measured by that caused by the glucose present, the reducing power was calculated as milligrams of 'maltose monohydrate' present, with a standard curve prepared with that sugar. The intensity of the colour is approximately proportional to the number of reducing groups present for maltose and greater molecules, including polysaccharides (Meyer, Noelting & Bernfeld, 1948; Meyer, van der Wyk & Feng, 1954), and thus a comparison with the total amount of carbohydrate present, as measured with the anthrone reagent, enabled the estimation of the average degree of polymerization of the polysaccharide, oligosaccharide and disaccharide present.

All determinations of mono-, di- or poly-saccharides on fasting intestinal contents, despite their richness in protein, have given values approaching zero. Glycerides and free fatty acids do not react with these reagents. Further, for each substance a characteristic site of absorption was found, beyond which the levels measured were essentially zero.

The accuracy of these methods was supported by the presence of 100% of the ingested disaccharide in the unabsorbed state in samples from the duodenum.

### *Calculation of the degree of absorption of carbohydrates*

In each experiment both the meal administered and the samples of intestinal contents obtained were analysed for polyethylene glycol and carbohydrate. The degree of absorption of sugar in each intestinal sample was calculated by the following formula:

$$\text{Absorption (\%)} = 100\left(1-\frac{S_i.P_m}{P_i.S_m}\right).$$

Absorption (%) means the amount of sugar absorbed, expressed as percentage of the original amount contained in the fraction of the meal which is present in the sample of intestinal contents collected. $S_i$, concentration of sugar (sum of monosaccharide and disaccharide) in the sample of intestinal contents; $P_i$, concentration of polyethylene glycol in this sample; $S_m$, concentration of sugar in the meal administered; $P_m$, concentration of polyethylene glycol in the meal.

## RESULTS

*Recovery of the test meal from the intestine.* The amount of intestinal contents obtained through the tubing varied between 50 and 400 ml./hr. Sampling was continued for 4 hr. after the ingestion of the test meal, and the amount of polyethylene glycol obtained through the tube during this time varied in individual experiments between 10 and 40% of the total amount ingested, but in most cases was between 20 and 25%. There was no correlation between the amount of polyethylene glycol recovered and the site of collection.

*Rate of emptying of the stomach.* Of the total amount of polyethylene glycol recovered in samples obtained from the duodenum, about 50% was obtained during the first hour of sampling, 40% during the second hour, 5–10% during the third hour and usually only a few per cent during the fourth hour. The passage of the test meal from the stomach to the duodenum thus seems to be essentially complete in about 3 hr. This is somewhat more rapid than in earlier experiments with a different test meal containing glucose, skim-milk powder and corn oil (Borgström *et al.* 1957). The rate of emptying of the stomach seemed not to be influenced by the particular carbohydrate present in the meal.

*Dilution of the test meal in the intestine.* The dilution of the test meal in the intestine, as calculated from the concentration of polyethylene glycol in the samples obtained, was two to five times in the upper part of the intestine. At lower levels of the intestine, the test meal was concentrated. These observations are in good accordance with the results obtained earlier (Borgström *et al.* 1957).

*Rate of passage through the small intestine.* When sampling was made at low levels in the intestine (200–220 cm. from the nose, i.e. the proximal part of the ileum), the distribution of the total amount of polyethylene glycol recovered between the different hours of sampling was more variable. Generally the amount of polyethylene glycol recovered seemed, however, to decrease during the third and fourth hours. Although the time taken for the whole meal to pass through the small intestine cannot be directly measured in these experiments, it can be concluded that after 4 hr. the major part of the meal has reached the ileum.

*Absorption of sucrose.* Sucrose absorption began in the lower jejunum and probably occurred chiefly in the ileum. No duodenal absorption was found (Fig. 1). The tube did not reach the ileum in the experiments where sucrose was fed. Nevertheless, the observation that more than 50% of ingested sucrose was not absorbed in passing through the jejunum, when added to the known excellent intestinal absorption of sucrose, is strong evidence for the major part of sucrose absorption occurring in the ileum.

The invertase activity of the contents of the jejunum, where sucrose absorption began, was low, usually below 1 unit/ml. (Fig. 2). During the first 2 hr. of absorption, these samples contained 10–70 mg. of sucrose/ml. (Fig. 2), which means that the invertase present could hydrolyse less than 5% of the sucrose/hr. In accordance with this the degree

of hydrolysis of sucrose in all samples was low, never exceeding 10 % (Fig. 3).

The fructofuranosyl group of sucrose is known to be extremely acid-labile, but the low degree of hydrolysis of sucrose in duodenal content, plus the calculated lack of absorption in these samples, indicated that no significant gastric hydrolysis had taken place. In several samples obtained from the stomach, despite the content having pH 3–4, as measured with a glass electrode, the hydrolysis of sucrose was less than 0·5 %.

*Absorption of maltose.* Maltose was not absorbed in the duodenum, but 50–70 % of that fed was absorbed in the jejunum, and the remainder in the proximal part of the ileum (Fig. 1).

The maltase activity of the intestinal contents was low, in most samples between 1 and 5 units/ml (Fig. 2). The concentration of maltose in the intestinal contents during the first 2 hr. of absorption was between 10 and 70 mg./ml. (Fig. 2). In agreement with this the degree of hydrolysis of maltose in the samples obtained from the region of most active absorption was low, at most about 11 %. In the samples obtained from the upper part of the ileum, where the major part of the maltose already had been absorbed (Fig. 1), the remainder had been hydrolysed to about 50 % (Fig. 3). This high degree of hydrolysis was, however, explained by the low maltose concentration and the relatively high maltase activity of these samples (Fig. 2), which is in sharp contrast with the conditions in the jejunum.

Under the assumption that maltose was hydrolysed by maltase present in the intestinal contents before absorption, the complete hydrolysis and

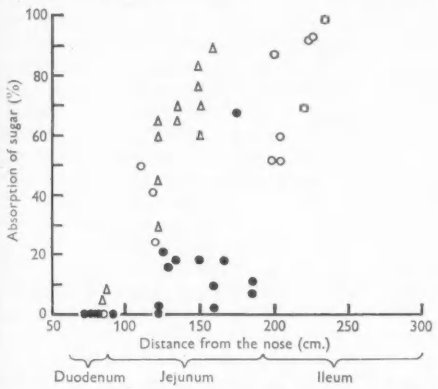


Fig. 1. Degree of absorption of sugar at different levels of the human small intestine after the ingestion of a test meal containing sucrose (●), maltose (○) or lactose (△), together with fat and protein.

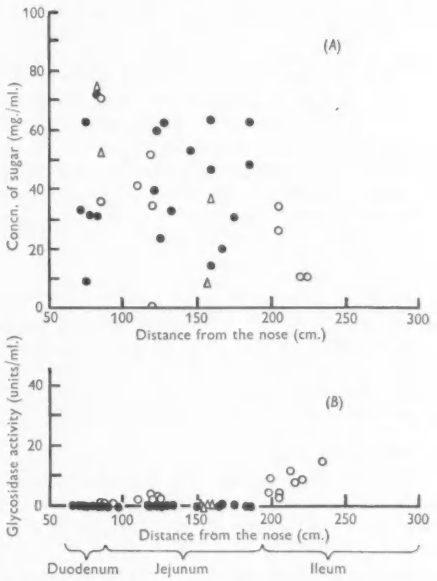


Fig. 2. Concentration of sugar (*A*) and glycosidase activities (*B*) in the intestinal contents during the first 2 hr. after the ingestion of a test meal containing sucrose, maltose or lactose, together with fat and protein. The figures for sugar concentration and enzymic activity may be compared, since 1 unit of glycosidase activity hydrolyses 1 mg. of disaccharide/hr. *A*: ●, Sucrose; ○, maltose; △, lactose. *B*: ●, Invertase; ○, maltase; △, lactase. Zero values for the lactase activity have been obtained also in samples from the duodenum and the proximal part of the jejunum, although these values could not be included in the Figure.

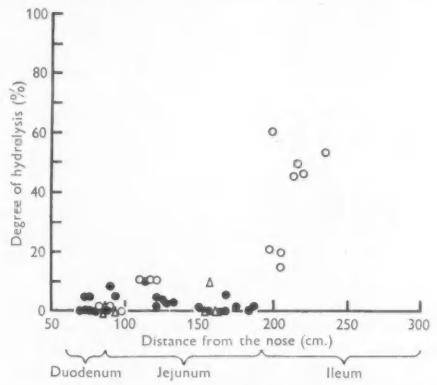


Fig. 3. Degree of hydrolysis of sucrose (●), maltose (○) and lactose (△) present in the intestinal contents at different levels of the human small intestine during absorption.

absorption of maltose would take more than 10 hr. after the sugar had reached the small intestine. It seems, however, that the major part of the maltose present in the test meal was absorbed about 4 hr. after the ingestion of the meal, suggesting adsorption in the disaccharide form. In portions of the test meal which reached the upper part of the ileum during the first hour, 70% of absorption of maltose had already occurred.

*Absorption of lactose.* Lactose was absorbed at a higher level in the small intestine than were the other two disaccharides investigated. The absorption of lactose began in the duodenum, and seems to have been completed before the meal reached the lower part of the jejunum (Fig. 1).

In spite of this the lactase activity of the intestinal contents was very weak, being below 0·5 unit/ml. in all samples (Fig. 2). The concentration of lactose in the intestinal contents during the first 2 hr. of absorption was between 8 and 74 mg./ml. (Fig. 2). The degree of hydrolysis of the lactose present was low in all samples (Fig. 3). The complete hydrolysis of the lactose by the lactase present in the intestinal contents would have taken at least 100 hr.; the lactose given in the test meal in fact seems to have been completely absorbed within 3–4 hr.

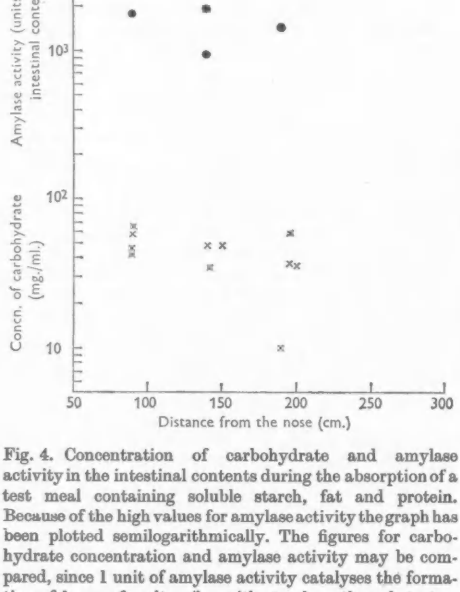


**Fig. 4. Concentration of carbohydrate and amylase activity in the intestinal contents during the absorption of a test meal containing soluble starch, fat and protein. Because of the high values for amylase activity the graph has been plotted semilogarithmically. The figures for carbohydrate concentration and amylase activity may be compared, since 1 unit of amylase activity catalyses the formation of 1 mg. of maltose/hr. with starch as the substrate.**

*Hydrolysis of starch.* In three subjects soluble starch was given in the test meal. The concentration of carbohydrate and the amylase activity found in the intestinal contents in these experiments are seen in Fig. 4. This graph has been plotted semilogarithmically, which was necessitated by the high amylase activity of these samples. The concentration of carbohydrate was between 10 and 65 mg./ml., and the amylase activity between 1000 and 9000 units/ml. In accordance with this the degree of hydrolysis was high. The average degree of polymerization of the oligosaccharides present was 2·1–2·4, i.e. at least between 60 and 90% of the amount of oligosaccharide present was in the form of disaccharide. Between 5 and 10% of the total amount of carbohydrate present in these samples was free glucose.

Because of the high amylase activity of these samples, the hydrolysis of starch may have proceeded in the flasks after sampling, although these were stored at 0°. On the other hand, since one unit of amylase will hydrolyse starch, forming 1 mg. of maltose/hr. at 37°, the amylase activity of the intestinal contents seems sufficiently high to hydrolyse the starch completely in the intestine within a few minutes. The hydrolysis of starch to disaccharides and oligosaccharides in the intestine thus is an extracellular process, in contrast with the hydrolysis of disaccharides.

## DISCUSSION

Both the low glycosidase activity measured and the low degree of hydrolysis of disaccharides in the intestinal contents during the digestion and absorption of the test meal indicate that the hydrolysis of disaccharides in the human intestine does not occur in the intestinal lumen, and that disaccharides are absorbed as such. It is well established that disaccharides infused into the systemic circulation are essentially quantitatively excreted by the kidneys (Verzár & McDougall, 1936). This observation, when added to the high glycosidase activity of mammalian intestinal mucosa (Dahlqvist, 1961*b*), seems to indicate that absorbed disaccharides are hydrolysed by intracellular mucosal enzymes and the monosaccharides pass into the portal system. We have observed (unpublished work) high glycosidase activities in a sample of human intestinal mucosa obtained from the lower part of the ileum. In agreement with the observations on the sites of disaccharide absorption this sample was rich in invertase and maltase activity, but poor in lactase.

Weld (1961) has described particles, named enterolipomicrons, that are present in the secretion

from Thiry loops of the duodenum or of the jejunum in dogs. These particles contain invertase, and Weld is of the opinion that they are the form in which the intestinal glycosidases are secreted. However, no comparison has been made between the amount of disaccharide which the enterolipomicrons are able to hydrolyse per time unit, and the amount of disaccharide actually absorbed by the intestine. On differential centrifuging of homogenates of intestinal mucosa Borgström & Dahlqvist (1958) found glycosidases in the microsome fraction. It seems probable that the enterolipomicrons, which have the same sedimentation properties as the microsomes (Weld, 1961), are formed from disintegrating cells in the intestinal juice.

Results in agreement with our conception of the intestinal glycosidases as intracellular enzymes have been obtained by studies of disaccharide absorption by hamster intestine *in vitro* (Miller & Crane, 1960; Crane, 1960). The experimental details have, however, not yet been published.

Studies *in vivo* and *in vitro* on rats have also indicated that the intestinal dipeptidases are localized inside the cells (Newey & Smyth, 1960). It is thus doubtful whether the 'succus entericus' contains any secreted digestive enzymes at all.

Weidenhagen (1932) suggested that the hydrochloric acid of the stomach was the most important factor in the hydrolysis of sucrose. The low degree of hydrolysis of sucrose in the intestinal contents, and the negligible hydrolysis of this disaccharide in the samples obtained from the stomach in our experiments, demonstrate, however, that the acidity of the stomach contents does not contribute to the hydrolysis of sucrose.

Ugolev (1960*a*, *b*) suggested that the hydrolysis of starch to disaccharides and oligosaccharides in the intestine was effected chiefly by amylase adsorbed to the intestinal wall, considerably higher activity being exerted in this way than when the enzyme is present in free solution. The name 'parietal digestion' was proposed for this mechanism.

In our experiments, however, the amylase activity in the intestinal contents was sufficiently high to hydrolyse the starch in a few minutes. The importance of 'parietal digestion' for the hydrolysis of starch thus could not be confirmed. The hydrolysis of starch to disaccharides and oligosaccharides, in contrast with the hydrolysis of the disaccharides, seems to occur entirely in the intestinal lumen.

Different disaccharides seem to be absorbed in different parts of the small intestine: lactose in the duodenum and the upper part of the jejunum, maltose in the jejunum and the upper part of the ileum, and sucrose in the lower part of the jejunum and in the ileum. The location of the different glycosidases along the human small intestine is not known. The absorption pattern found in these experiments parallels the localization of the different disaccharides over the length of the intestine in the adult pig (Dahlqvist, 1961*b*). The complete absorption of lactose in the proximal part of the intestine (in fact lactose is absorbed at the same level in the small intestine as is free glucose, Borgström *et al.* 1957) seems especially remarkable, since lactose is believed to be utilized with difficulty by adults (Day & Pigman, 1957). The amount of lactose given in the test meal in our experiments corresponds to somewhat more than 1 l. of cow's milk, and seems to be absorbed rapidly, although the total rate of absorption could not be directly measured.

Most of our previous knowledge of the absorption of lactose is, however, based upon experiments with animals, mostly rats (Fischer & Sutton, 1949; Day & Pigman, 1957).

## SUMMARY

1. Human subjects have been given the disaccharides sucrose, maltose or lactose, or the polysaccharide starch, in a test meal containing protein, homogenized fat and a non-absorbable reference substance, polyethylene glycol. Intestinal contents were collected by intubation, and the amount of hydrolysis and absorption of carbohydrates were calculated and compared with the carbohydrase activity of the samples.

2. The limited hydrolysis of disaccharides in the intestinal contents, and the extremely low glycosidase activities relative to the calculated amount of carbohydrate absorbed, indicated that the majority of disaccharides were absorbed unhydrolysed, followed by subsequent intracellular hydrolysis.

3. Lactose was absorbed in the duodenum and proximal jejunum, maltose in the jejunum and proximal ileum and sucrose in the distal jejunum and in the ileum.

4. Ingested starch was rapidly hydrolysed to di- and oligo-saccharides by amylase present in the intestinal contents.

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# The Precipitation of Polyanions by Long-Chain Aliphatic Ammonium Salts

## 5. THE INFLUENCE ON PRECIPITATION OF CHANGES IN THE AMINE STRUCTURE*

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Several authors have shown that many polyanions are precipitated from aqueous solution by organic cations containing a long chain of $CH_2$ units, e.g. cetylpyridinium and cetyltrimethylammonium (for a review see Scott, 1960). Polyanions that take part in this reaction include polyacrylic acid, negatively charged proteins, polyuronides, sulphated polysaccharides, polyphosphates, nucleic acids and borate complexes of neutral polysaccharides. It was concluded (Scott, 1956, 1960) that the reaction is probably general for all water-soluble polyanions.

Scott (1955*a*) demonstrated that the insoluble complexes were salts of the polyanion and the aliphatic ammonium cation. The stoicheiometric combination of, e.g., cetylpyridinium with various polyanions was shown to be of use in the estimation of their acidic groups (Scott, 1955*a*). The complexes are very insoluble (less than 1 mg./100 ml.) and small amounts of polyanion may be precipitated quantitatively from dilute solutions: a useful step in the isolation of polyanions from biological materials, since they can be easily recovered from the complex (Scott, 1960).

The mechanism of the precipitation reaction has been discussed (Scott, 1956) and it was suggested that there were two alternatives, depending on whether the quaternary ammonium ion was considered to react as micellar aggregates or as single ions. The available evidence was insufficient to allow a conclusion to be drawn. The emphasis in this paper is on the influence on the reaction of changes in the structure of the amine. As a result of the investigation it is possible to propose a mechanism of precipitation. Apart from the intrinsic interest of the reaction, it is desirable that it should be better understood so that improvements in the practical application may be made. A preliminary report of some of the results has been given (Scott, 1961).

## MATERIALS AND METHODS

*Abbreviations.* $R^+$, Organic cation; $M^+$, inorganic cation; $P^{z-}$, polyanion with valency $z$.

### *Aliphatic ammonium salts*

Unbranched primary amines with chain lengths $C_6$–$C_{18}$ were obtained from L. Light and Co. Ltd. They were converted into the hydrochloride without further purification.

* Part 4: Antonopoulos, Borelius, Gardell, Hamnström & Scott (1961).

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Table 1. *Precipitation of heparin and hyaluronate by primary amine hydrochlorides* $C_nH_{2n+1}NH_3Cl$

Heparin and hyaluronate in aqueous solution were titrated with solutions of primary ammonium salt to the flocculation end point (see Materials and Methods section). The values marked * are of low precision because of the small quantities of titrant added (0·02 ml.), and are upper limits.

| | $C_n$ | | | | | |
|---|---|---|---|---|---|---|
| | $C_6$ | | $C_8$ | | $C_{10}$ | |
| Sodium heparinate | | | | | | |
| Titration volume at the end point | 0·68 | 3·3 | 0·52 | 2·58 | 0·52 | 2·53 |
| Normality of heparin at the end point | 0·0076 | 0·00158 | 0·01 | 0·002 | 0·0098 | 0·0025 |
| Normality of amine at the end point | 0·265 | 0·245 | 0·031 | 0·029 | 0·019 | 0·005 |
| (Normality of amine) – (normality of heparin) = excess amine concentration | 0·257 | 0·244 | 0·030* | 0·027 | 0·009* | 0·0025 |

| | $C_n$ | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $C_8$ | | $C_{10}$ | | $C_{12}$ | | $C_{14}$ | |
| Sodium hyaluronate | | | | | | | | |
| Titration volume at the end point | 0·6 | 2·8 | 0·52 | 2·58 | 0·54 | 2·58 | 0·56 | 2·56 |
| Normality of hyaluronate at the end point | 0·004 | 0·00071 | 0·0048 | 0·00097 | 0·0047 | 0·00097 | 0·0045 | 0·00098 |
| Normality of amine at the end point | 0·166 | 0·18 | 0·020 | 0·0165 | 0·0072 | 0·004 | 0·0050 | 0·00122 |
| (Normality of amine) – (normality of hyaluronate) = excess amine concentration | 0·162 | 0·18 | 0·015* | 0·0155 | 0·0025 | 0·003 | 0·0005 | 0·0002 |

$C_6$, $C_8$, $C_9$ and $C_{10}$ amines were titrated directly with 2N-HCl in water, by using a Cambridge pH meter, to a pH between 6·3 and 6·8. Amines with $C_{12}$, $C_{14}$ and $C_{16}$ chains were dissolved in ether, and HCl gas from a generator was bubbled through the solution. The solid hydrochloride separated out as a white deposit, which was washed with ether and recrystallized from water. Secondary amines, di-*n*-propylamine, di-*n*-amylamine and di-*n*-hexylamine were also obtained from L. Light and Co. Ltd. They were titrated in aqueous solution with 2N-HCl to pH 6·5.

The quaternary alkyltrimethylammonium salts were gifts from Imperial Chemical Industries Ltd. and Armour Hess Chemicals Ltd., 6 Arlington St., London, S.W. 1. $C_8$, $C_{10}$, $C_{12}$ and $C_{14}$ were from the Imperial Chemical Industries Ltd. specimen collection, and were obtained as bromides. $C_{16}$ and $C_{18}$ were Arquad preparations from Armour Hess Chemicals Ltd. and were obtained as chlorides. All the compounds were used without further purification.

Didecyldimethylammonium bromide was obtained from the British Hydrological Corp., Colloidal Works, High Path, Merton, London, S.W. 19.

Stock solutions were prepared in water and were analysed for N content by the Kjeldahl method, performed according to Fawcett (1954) except that in general a digestion time of 1 hr. was used. Concentrations calculated from the N content were used as the basis for all calculations.

### *Polyanions*

*Hyaluronic acid.* This was prepared from the exudate of a human myxoma according to the method of Scott (1960).

*Heparin.* This was commercial, 100 units/mg., obtained as an aqueous solution (50 mg./ml.) from Paines and Byrne Ltd., Pabyrn Laboratories, Greenford, Middlesex.

*Keratosulphate.* This was from nucleus pulposus, a gift of Dr Sven Gardell.

*Sodium alginate (B.P.C.) and pectin (apple 200 grade).* These were commercial samples from British Drug Houses Ltd., used without further purification. The pectin contained 8% methoxyl and 20% moisture.

All the above polyanions were sodium salts.

### *Titration–precipitation*

Aqueous solutions of the polyanion containing 0·5–3 mg./ml. were incubated at 30° in a water bath in 5 ml. tubes. The aliphatic ammonium salt solution was added dropwise from a microburette, and mixed with the polyanion solution by gently rotating the tube. The 'end point' occurred when all the polyanion–organic cation complex had flocculated, leaving a clear solution between the particles (or, rarely, when there was no further increase in turbidity with addition of amine solution). Each titration was performed at least twice. The concentrations of the amine solutions varied from 1M for hexylammonium chloride to 0·01M for tetradecylammonium chloride, with the general aim of preventing too great a variation in titration volume throughout the series.

## RESULTS

### *Titration–precipitation of polyanions*

The precipitate of each polyanion forms in the presence of decreasing amounts of aliphatic ammonium salt as the chain length in the latter increases (Table 1). When a paraffin chain with more than 12 or 14 $CH_2$ units is involved, precipitation occurs when there is practically a one-to-one ratio of aliphatic ammonium cations with the anionic groups of the polymer. That is, the quantity of aliphatic ammonium salt required to produce a flocculent precipitate depends on the total quantity

of polyanion in the solution. The situation is quite different when the chain is of six $CH_2$ units. The formation of a flocculent precipitate then depends on the concentration of the amine cation, which is much higher (about $10^3$ times) than that of the polyanion. There is no equivalence between polyanion and amine. If identical amounts of polyanion are titrated in different volumes of solution, the quantity of hexylammonium ($C_6$) ion needed to produce a flocculent precipitate is proportional to the total volume. The behaviour of amines with single chains of 8, 10 or 12 $CH_2$ units is intermediate between the two extremes, with a steady and marked trend towards stoicheiometric combination as the carbon chain is lengthened. These points are illustrated in Table 1, which gives results of typical experiments involving heparin and hyaluronate.

If the amine cation is assumed to combine stoicheiometrically with the polyanion in the precipitate at the end point, throughout the series $C_6$–$C_{16}$ (which is known to be the case, e.g. with cetylpyridinium), the quantity combined can be subtracted from the total amine in the solution to give the concentration of free amine, which will be called the excess amine concentration. It is expressed in normality and is nearly independent of polyanion concentration in these experiments. For the purpose of the calculation the polyanions were assumed to possess the structures generally attributed to them (e.g. as shown in Whistler & Smart, 1953).

Table 2 shows values of the excess amine concentrations, for a series of aliphatic ammonium ions with one or two paraffin chains, in the presence of several polyanions. With heparin and alginic acid the values for amines with more than 10 or 12 $CH_2$ units became so small as to be not measurable.

Fig. 1 shows that the plot of the logarithm of the excess amine concentration against chain length ($C_n$) of the amine is a straight line, for all the polyanions tested in this way.

In a similar series of experiments with unneutralized polyacrylic acid as the sole polyanion, Saito (1955) showed that the plot of the log of the total amine concentration against $n$ was a straight line, in the region of shorter chain lengths, but that the slope became very much less as the chain length increased to $C_{12}$ and above. This is mainly because the total amine concentration at flocculation point is partly determined by the polyanion concentration and is much less a property of the amine itself. When Saito's results are recalculated to give excess amine concentrations, the line is straight at all chain lengths, as shown in Fig. 1.

The difference between the excess amine concentrations of primary and quaternary amines with the same paraffin chain is very considerable. It was observed that the precipitates obtained with the primary amines were almost always clean and flocculent, with a clear end point, whereas the quaternary amines more frequently gave a slightly diffuse end point, especially when the chain length was $C_{12}$ or less. Keratosulphate, which is not easily titrated by quaternary amines (e.g. cetylpyridinium) because it gives a diffuse end point, and also because the precipitate may redissolve in the presence of a large excess of quaternary amine, was easily titratable with the longer-chain primary amines, such as tetradecylammonium chloride, to give a very good flocculent precipitate. There was

Table 2. *Excess amine concentrations of aliphatic ammonium salts in the presence of several polyanions*

The excess amine concentrations, expressed in normalities, of amine salts with aliphatic chains R, are in each case averages of several experiments of the type presented in Table 1. Where the appropriate value is not entered, it was not measured. Excess amine concentrations are included for keratosulphate, although it is not a well-characterized polysaccharide, because of its interesting behaviour in these systems (see the Results section).

| | | R | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Precipitant | $C_3H_7$ | $C_5H_{11}$ | $C_6H_{13}$ | $C_8H_{17}$ | $C_9H_{19}$ | $C_{11}H_{21}$ | $C_{12}H_{25}$ | $C_{14}H_{29}$ |
| Sodium pectinate | $RNH_3^+$ | — | — | — | 0·25 | — | 0·036 | 0·006 | — |
| Sodium hyaluronate | $RNH_3^+$ | — | — | 1·0 | 0·15 | — | 0·015 | 0·002 | 0·0002 |
| | $RNMe_3^+$ | — | — | — | — | — | 0·3 | 0·016 | 0·005 |
| | $R_2NH_2^+$ | > 0·75 | > 0·2 | > 0·1 | — | — | — | — | — |
| Sodium alginate | $RNH_3^+$ | — | — | — | 0·036 | — | 0·004 | — | — |
| Sodium heparinate | $RNH_3^+$ | — | — | 0·25 | 0·028 | 0·005 | 0·003 | — | — |
| | $R_2NH_2^+$ | > 0·8 | > 0·2 | Turbidity at 0·125 | — | — | — | — | — |
| | $RNMe_3^+$ | — | — | — | 0·27 | — | 0·04 | 0·0055 | — |
| | $RNH\,Me_2^+$ | — | — | — | — | — | 0·022 | — | — |
| Sodium keratosulphate | $RNH_3^+$ | — | — | 0·5 | 0·149 | 0·07 | 0·04 | — | — |
| | Didecyldimethylammonium bromide | — | — | — | — | — | 0·001 | — | — |

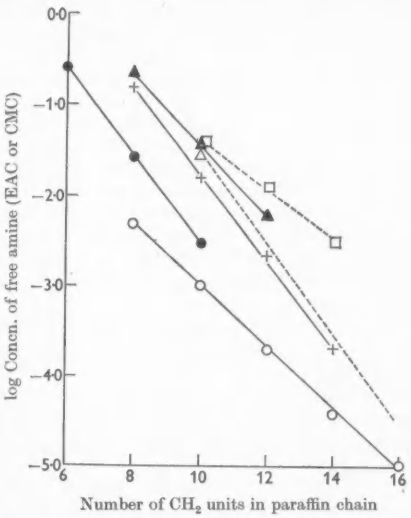


Fig. 1. Excess amine concentrations (EAC) and critical micellar concentrations (CMC) of normal aliphatic amines plotted logarithmically against the number of $CH_2$ units in the aliphatic paraffin chain. —, Excess amine concentrations, which are of primary amines only; - - -, critical micellar concentrations; ▲, sodium pectinate; +, sodium hyaluronate; ●, sodium heparinate; ○, polyacrylic acid; □, primary aliphatic ammonium chlorides in water; △, quaternary trimethylammonium bromides in concentrated sodium chloride solution. The points on the polyacrylic acid line are calculated from the data of Saito (1955). The CMCs in sodium chloride solution are based on data of Corrin & Harkins (1947), and of Phillips (1955). The CMCs in water are according to Klevens (1948).

no apparent tendency for the precipitate to redissolve in the presence of excess of tetradecylammonium chloride. Keratosulphate is also precipitated cleanly by didecyldimethylammonium bromide.

## DISCUSSION

### *Precipitation by micelles versus precipitation by single ions*

The consideration of the mechanism of the precipitation of polyanions by organic cations in the absence of added electrolyte is complicated by the fact that amphipathic organic cations can react in two different states, as single ions or as micelles, and both are often present in solution together. It is possible to explain many of the experimental observations by postulating that either single ions or micelles are involved, to the exclusion of the other, and there is a third possibility, which cannot be totally excluded, that both may be involved simultaneously.

It was suggested by Teryama (1955) that the precipitate that he obtained on mixing equivalent amounts of cellulose sulphate and cetyltrimethylammonium bromide was an example of combination between oppositely charged colloidal particles, such as he had observed between the polycation chitosan and several polyanions. The positively charged micelle of cetyltrimethylammonium bromide was thought to be analogous to the positively charged polymer of chitosan. Scott (1955*b*) suggested that single amphipathic ions might be the precipitant, and later concluded (Scott, 1956) that polyanions could be precipitated by quaternary ammonium ions at concentrations so small that micelles were unlikely to be present.

In the present paper it is shown that polyanions are precipitated by organic cations that are not normally regarded as forming micelles, e.g. hexylammonium, and by octylammonium and decylammonium at concentrations far below those in which micelles are known to be present.

If the precipitation of polyanions depends on the existence of micelles, a given organic cation should not be capable of precipitating polyanions until it is above the concentration at which micelles are formed (the 'critical micellar concentration'). This implies that the excess amine concentration of an organic cation should be constant, irrespective of the nature of the polyanion. Table 1 shows that this is not so: there is a very marked dependence of this value on the structure of the polyanion.

Further, if micelles of organic cation are the effective precipitants, the graph of the log of the excess amine concentration against $n$, the number of carbon atoms in the organic cation aliphatic chain, should be parallel to, if not coincident with, the graph of log of critical micellar concentration against $n$. Fig. 1 shows that the two lines are widely separated and of quite different slope.

It is therefore concluded that micelles do not participate in the precipitation reaction in the absence of added electrolyte. It remains to be shown that the experimental observations can be explained on the basis of precipitation by single ions.

### *Flocculation end point*

The occurrence of a flocculation end point may be accounted for by applying the Law of Mass Action to the equation

$$P^{z-}zM^+ + zR^+ \rightleftarrows P^{z-}zR^+ + zM^+, \qquad (1)$$

which gives

$$\frac{[P^{z-}zR^+]}{[P^{z-}zM^+]} = K\frac{[R^+]^z}{[M^+]^z}. \qquad (2)$$

The left-hand expression will be equated with the degree of precipitation, $D$. If arbitrary values of $K$ are chosen (the precise values are immaterial) and

the ratio $[R^+]/[M^+]$ is calculated for 1, 50 and 99 % precipitation, for even quite small values of $z$ ($\geqslant$ 10–20), $D$ increases from very small values to almost 100 % for a very small change in the value of $[R^+]/[M^+]$. On either side of a very small range of values of $[R^+]/[M^+]$, the polyanion exists almost entirely as $P^{z-}zM^+$ or $P^{z-}zR^+$. The flocculation end point, which is sharp, is the visible manifestation of the sudden appearance of $P^{z-}zR^+$ in large quantity.

Equation (2) predicts an influence of molecular weight on precipitation by organic cations, since $z$ is directly proportional to the molecular weight, if $P^{z-}$ is a homogeneous polymer.

*Free energy of formation of* $P^{z-}zR^+$

The relationship

$$\Delta F = -\boldsymbol{RT} \ln K \tag{3}$$

may be applied to equation (1) where $K$ is the equilibrium constant and $F$ is the free-energy change involved. Certain assumptions are made, which are mentioned in this discussion.

From (2), ignoring activity coefficients:

$$K = \frac{[P^{z-}zR^+]\,[M^+]^z}{[P^{z-}zM^+]\,[R^+]^z} = D\frac{[M^+]^z}{[R^+]^z}. \tag{4}$$

If two organic cations, $R_1^+$ and $R_2^+$, are chosen:

$$\frac{K_{R_1^+}}{D_1} = \frac{[M^+]_1^z}{[R_1^+]^z} \tag{5}$$

and

$$\frac{K_{R_2^+}}{D_2} = \frac{[M^+]_2^z}{[R_2^+]^z}. \tag{6}$$

From (5) and (6), if $D_1 = D_2$, the degree of precipitation at the flocculation point:

$$\frac{K_{R_1^+}}{K_{R_2^+}} = \frac{[M^+]_1^z\,[R^+]^z}{[M^+]_2^z\,[R_1^+]^z}. \tag{7}$$

Substituting from (3), and if $[M^+]_1 = [M^+]_2$:

$$\frac{\Delta F_{R_2^+} - \Delta F_{R_1^+}}{\boldsymbol{RT}} = z \ln \frac{[R_2^+]}{[R_1^+]}. \tag{8}$$

$[R_1^+]$ and $[R_2^+]$ are equivalent to the two excess amine concentrations ($EAC_1$ and $EAC_2$) at flocculation point, therefore:

$$\Delta F_{R_1^+} - \Delta F_{R_2^+} = z\boldsymbol{RT} \ln \frac{EAC_1}{EAC_2}. \tag{9}$$

The ratio of the two excess amine concentrations is approximately 10 (Table 1). $\Delta F_{R_1^+} - \Delta F_{R_2^+}$ is the free-energy change associated with the transfer of $2z$ $CH_2$ units from water to the complex, and therefore the average value of the free-energy change associated with two $CH_2$ units is:

$$\frac{\Delta F_{R_1^+} - \Delta F_{R_2^+}}{z} = 2{\cdot}3\ \boldsymbol{RT}.$$

This is in approximate agreement with the value of 2·7 $\boldsymbol{RT}$ obtained from the transfer of two $CH_2$ units from water to oil and in good agreement with 2·4 $\boldsymbol{RT}$, the value obtained by Phillips (1955) for the transfer of two $CH_2$ units from water to a detergent micelle.

It is an approximate value since, although the volumes of titration at flocculation point were generally not very different from $R_1^+$ to $R_2^+$ (by taking more concentrated solutions of organic cation as the excess amine concentration increased), $[M^+]_1$ was seldom exactly the same as $[M^+]_2$. It also assumes that $D$ is the same at the flocculation point for different organic cations.

Conversely, if the 'oily' nature of the polyanion–organic cation complex is assumed, then

$$\Delta F_{R_1^+} - \Delta F_{R_2^+}$$

would be expected to be constant, whereupon (9) becomes

$$\ln \frac{EAC_1}{EAC_2} = \text{a constant},$$

which is equivalent to the straight-line relationship shown in Fig. 1.

This argument predicts the constancy of the excess amine concentration of a polyanion if $[M^+]$ is constant, at any given ratio of $[P^{z-}zR^+]/[P^{z-}zM^+]$, i.e. at a given percentage precipitation. In these circumstances the excess amine concentration is a constant of the polyanion, and could in theory be used in the characterization or isolation of the polyanion.

*Electrostatic factor in the formation of* $P^{z-}zR^+$

The formation of detergent micelles in solution depends on a balance between two opposing factors: (1) the alignment together of hydrocarbon chains, in order to minimize their area of contact with water; (2) the repulsion between cationic groups at the end of the hydrocarbon chains. The repulsive force is considerably lessened if the cationic groups are screened from each other by a high electrolyte concentration, and it has been experimentally observed (Corrin & Harkins, 1947) that the critical micellar concentration is much lower in salt solutions than in water. The slope of log critical micellar concentration against $n$, the number of $CH_2$ units in the hydrocarbon chain, has been given as 0·5 in concentrated electrolyte solution by Phillips (1955) based on the data of Taylor, as compared with 0·3 in water. A line has been plotted in Fig. 1, with the value for decyltrimethylammonium bromide (Corrin & Harkins, 1947) in concentrated sodium chloride solution as the fixed point for a line of slope 0·5. A second line based on the findings of Klevens (1948) for primary amine hydrochlorides in water is also plotted, showing a much smaller slope. The line of log excess amine concentration has about the same slope as the line of log critical micellar concentration in concen-

trated electrolyte solution. The screening action of the concentration of negative charges on the polyanion is analogous to the screening effect of a high electrolyte concentration. The concentration of negative charge of a polyanion is, however, much higher than that of almost any attainable electrolyte solution, and the screening action of a polyanion such as heparin is probably equivalent to that of a 'swamping excess' of electrolyte, i.e. the maximal effect is being exerted. The slope of log excess amine concentration should be dependent on charge density, which determines the screening power. There is a suggestion that the slope of the pectin line is less than that of the heparin line (Fig. 1), corresponding to a decrease in the screening action. The slope of log excess amine concentration for polyacrylic acid is only slightly greater than that for log critical micellar concentration in water, presumably reflecting the very low charge density, and therefore low screening capacity, of unneutralized polyacrylic acid. The generally low values of the excess amine concentrations with polyacrylic acid, as compared with carbohydrate polyanions, are probably due to the fact that organic cations can cohere to the main polymer chain of polyacrylic acid, which is of a hydrocarbon nature, as well as to each other. The hydrophilic carbohydrate polymers do not offer comparable absorption sites.

*Qualitative hypothesis of the precipitation process*

The implications of the single-ion mechanism can be qualitatively visualized by considering the sequence of events during the titration of a polyanion in aqueous solution with an organic cation (Scott, 1960). In the initial stages the organic cation is distributed throughout the solution, some of it being associated with the anionic groups of the polymer. The percentage of organic cation bound to the polyanion is determined by the affinity of the cation for the anionic groups. With increasing concentrations of organic cation, more is associated with the polyanion until 'bound' organic cation is likely to find itself close to another organic cation, similarly bound. The paraffin chains of both ions can then lie together or 'coalesce' with the liberation of free energy, which results in a big apparent increase in affinity of the organic cation for the polyanion. The apparent affinity of organic cation for the polyanion added at this point is greater than the affinity of organic cation at the outset, when there was no possibility that this type of co-operative binding could occur. At this stage the percentage of organic cation associated with the polyanion begins to increase. The amount of overlap of the paraffin chains with each other increases as more organic cation is bound, until organic cation must be bound at sites that are surrounded by previously bound organic cation. At this stage there is a maximum value of the affinity of organic cation for the polyanion. When all anionic sites are occupied by organic cations, the charge of the polyanion is neutralized. The solution contains polyanion–organic cation complexes, which are outwardly similar to oil droplets, with an inner core of polyanion that may be almost completely hidden from the water. It is then possible for these neutral hydrophobic particles to coalesce to form a separate phase. The structure of the neutral polyanion–organic cation complex is analogous to that of the micelle of organic cation that is present in aqueous solution. In the micelle the ionized groups are turned outwards to the water, with the hydrocarbon chains turned inwards away from the water. The polyanion–organic cation complex is a 'reversed' micelle.

The main driving force leading to the formation of the polyanion–organic cation 'reversed' micelle and the ordinary micelle is the same. The presence of a hydrocarbon chain in water breaks up the water structure, and with it many hydrogen bonds. There is therefore a tendency for the water to expel the hydrocarbon chain, or, failing this, to reduce the area of contact to a minimum. This is done if the hydrocarbon chains can be made to lie together, as in micellar structures.

The following points arise from a consideration of this mechanism:

(*a*) The degree of overlap between adjacent organic cations depends on the length of the paraffin chain in the cation. A very long chain will be able to 'coalesce' to a very large extent with a neighbouring, similar chain. The free energy liberated is considerable, and, therefore, the apparent affinity for the polyanion is greater than if the chain length was shorter. Thus the partition of single organic cations between the solution and polyanion is in favour of the polyanion, i.e. there is a low concentration of free (unbound) organic cation. If a shorter chain is involved, the degree of overlap is less, the affinity is therefore less, and the partition of organic cation between solution and polyanion is more in favour of the solution. This is illustrated in Table 1. The excess amine concentrations (= free organic cation) of the long-chain organic cations are much less than those of short-chain organic cations.

(*b*) There should be considerable overlap of adjacent bound organic cations when the anionic sites of a polymer are close together. By the reasoning of the previous paragraph, the affinity of organic cations for a polyanion of high charge density should be higher than for one of low charge density, i.e. there should be a low excess amine concentration for polyanions of high charge density. Table 1 shows that this is true. In pectin

there is approximately one anionic group per four sugar units, in hyaluronic acid one per two sugar units, in alginic acid one per one sugar unit, and in heparin one and a half (or more) anionic groups per sugar unit. The excess amine concentrations decrease in the order pectin > hyaluronate > alginate > heparin, for any particular organic cation. The result obtained by this reasoning is the same as that arrived at earlier, on the basis of the electrostatic screening action of the anionic groups of the polymer. It is because the screening power and the degree of overlap of adjacent bound organic cation are functions of the same variable (i.e. the distance between adjacent anionic groups).

(*c*) If two short chains, each containing $n$ $CH_2$ units, are present in an organic cation, there is a smaller possibility of overlap with similar organic cations bound on adjacent sites of a polymer than if one chain of $2n$ $CH_2$ units is involved. It is probable that the two chains of the dialkylammonium cation lie together, producing a more globular shape, without the necessary length to overlap with adjacent organic cations. Thus the dipropylammonium has a much higher excess amine concentration than hexylammonium, and even diamylammonium is not as good a precipitant as hexylammonium (Table 2). However, when each chain in the dialkylamines is considerably increased, the total bulk of $CH_2$ units presumably offsets to some extent the lack of length in the paraffin chain, and overlap of the more rigid globule is possible on two sides.

(*d*) The organic cation–polyanion affinity is affected by the nature of the cationic group of the organic cation. It seems from the experiments in Table 1 that the primary ammonium cation has a greater affinity for anionic groups than has the quaternary ammonium ion. The increased binding of primary ammonium ions, as compared with quaternary ammonium ions, is approximately equivalent to the addition of two $CH_2$ units to the quaternary ammonium ion paraffin chain. The greater affinity of primary ammonium ions for anionic groups is presumably responsible for the sharper and clearer end points observed with, for example, tetradecylammonium chloride as compared with cetylpyridinium chloride.

An examination of the hypothetical structures of the precipitates produced according to either the micellar or single-ion mechanisms suggests that they are probably identical. In both cases polyanion is embedded in a matrix of paraffin chains. By the micellar mechanism the paraffin chains align together before combination with the polyanion, whereas by the single-ion mechanism alignment takes place partly during combination, and then by aggregation of the polyanion–organic cation particles. However, the process by which the end is reached may be important in practice, and it seems unnecessary to postulate that micelles are required for the precipitation of polyanions from aqueous solution. This does not mean that precipitation by micelles is not possible, but it is suggested that precipitation by single ions is the more effective, and will usually occur before conditions for micellar precipitation are ripe. This is especially true of precipitation by primary ammonium salts.

## SUMMARY

1. The precipitation of alginate, heparin, hyaluronate, keratosulphate and pectinate from aqueous solution by aliphatic ammonium ions containing paraffin chains of from $[CH_2]_6$ to $[CH_2]_{18}$, and with several types of cationic group, has been studied.

2. The efficiency of precipitation increases in a regular and marked manner with increase of chain length. This effect can be accounted for if the insoluble complexes are assumed to be 'oily' particles, and a free-energy change of about $1{\cdot}2\ RT$ is associated with the transfer of $CH_2$ from the aqueous phase to the particle.

3. The concentration of organic cation required to precipitate a polyanion depends on the structure of the polyanion. Polyanions with high charge densities are precipitated more readily than less-densely charged polyanions.

4. It is concluded that precipitation of polyanions by primary ammonium salts is unlikely to be a micellar phenomenon. The occurrence of a flocculation end point can be accounted for by applying the Law of Mass Action to the reaction equation, involving single ions.

5. A qualitative hypothesis of the reaction mechanism is proposed.

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# Phosphoinositides

## 4. INOSITOL LIPIDS OF SCYLLITOL-CONTAINING TISSUES*

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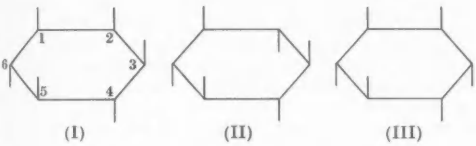
*myo*Inositol (I) is the only isomer of inositol yet found in phospholipids. *scyllo*Inositol (scyllitol, II) has long been known to occur free in the tissues of elasmobranch fishes (Staedler & Frerichs, 1858), in the acorn (Vincent & Delachanal, 1887) and in various other plants (Müller, 1907, 1912; Goodson, 1920; Hann & Sando, 1926). A search has been made, therefore, in two of these tissues, the acorn and the liver of *Raia clavata* (the common ray), for lipids containing *scyllo*inositol.




(I) (II) (III)

## MATERIALS AND METHODS

*Materials.* Liver from *Raia clavata* was obtained from the Marine Biology Laboratory, Plymouth. Batches of 500 g. were packed in ice for transport to Birmingham. Two batches of acorns from the common oak (*Quercus robur*) were extracted. The first batch was picked in early September from local trees. The acorns were not fully ripe and were green. The second batch was collected from the ground in December. They were dark brown and some had begun to germinate. The two batches behaved similarly in the experiments reported below.

Pure *scyllo*inositol, prepared by the reduction of *myo*-inosose-2 according to Raymond (1957), was a gift from Dr R. B. Ellis.

*Ionophoresis.* Separations of *scyllo*- and *myo*-inositol by paper chromatography in methanol–water mixtures have been described by Fleury, Courtois & Malangeau (1953). Angyal, McHugh & Gilham (1957) suggested phenol–water (4:1, w/w). In the present work, authentic samples of the two isomers failed to separate in these solvents, but ionophoresis in borate solutions (Foster & Stacey, 1953) gave good separations. Strips of Whatman no. 1 paper (50 cm. × 7 cm.) held in position by glass plates were used. The electrolyte was 0·012M-sodium tetraborate and a potential of 950v was applied for 3 hr. Under these conditions the relative mobilities towards the cathode were: *scyllo*inositol 15 cm., *myo*inositol 4 cm. The best results were obtained by applying small volumes (approx. 0·01 ml.) of the inositol solutions to the paper strips, so that spots of liquid no bigger than 5 mm. in diameter were produced.

The silver nitrate method of Trevelyan, Procter & Harrison (1950) was used for detection of the spots. In the presence of borate, development of the spots is slower than usual. For this reason, the use of borate solutions stronger than 0·012M was avoided.

*Other analytical methods.* Methods for the determination of phosphate and for the chromatography of phospholipid constituents have been described by Hawthorne & Hübscher (1959).

## EXPERIMENTAL

### *Preparation of non-lipid inositols from acorns*

Since the method used involves a hydrolysis, the products will include free inositols and inositols liberated from any water-soluble compounds such as phosphate esters. Two methods of preliminary treatment were used. In the first, the minced acorns (880 g.) were homogenized for 1 min. in a high-speed mixer with 1·2 l. of acetone. After 30 min. at room temperature the solvent was filtered off and distilled *in vacuo* until only a watery extract remained. Conc. HCl (2 vol.) was then added and the mixture refluxed for 18 hr.

In the second method, 500 g. of minced acorns was homogenized with 1 l. of 0·6N-$HClO_4$. After 18 hr. at room temperature, the mixture was centrifuged and the solid re-extracted in the same way. The combined supernatants were filtered by gravity and neutralized with 5N-KOH. The precipitated $KClO_4$ was filtered off and the solution refluxed with 2 vol. of conc. HCl as above.

After refluxing, the mixture in each case was cooled and filtered to remove the copious black precipitate. The clear brown filtrate was concentrated *in vacuo* to a syrup, to remove most of the HCl, then redissolved in 50 ml. of water and decolorized with charcoal. The filtrate after charcoal treatment was mixed with excess of Dowex 50 (100–200 mesh; $H^+$ form) and Dowex 2 (100–200 mesh; $OH^-$ form). The resins were removed by filtration and the colourless ion-free liquid was tested for the inositols as described below.

### *Characterization of the non-lipid inositols of acorn*

The colourless liquid obtained by the method described above was evaporated on a water bath to a volume of 5 ml., and while it was still hot an equal volume of hot absolute ethanol was added. After an hour at 4°, the mixture deposited white crystals. These melted at a temperature above 300° and so could have contained little

* Part 3: Kemp, Hübscher & Hawthorne (1961).

*myo*inositol (m.p. 225°). *scyllo*Inositol melts with decomposition at 352° (Raymond, 1957).

Ionophoresis of the concentrated aqueous solution (0·01 ml./spot) from another batch of acorns showed a strong *scyllo*inositol spot and a much fainter *myo*inositol spot. To judge from the size and intensity of the spots there was roughly ten times as much *scyllo*inositol as *myo*-inositol.

### *Preparation of free inositols from* Raia clavata *liver*

In this case no hydrolysis step was included, so that the products represent free inositols only.

Again, two preliminary methods were used. In the first, the liver (255 g.) was homogenized with 500 ml. of water in a high-speed mixer. The homogenate was filtered overnight by gravity and the material on the filter paper re-extracted twice with 500 ml. of acetone each time. Acetone and water extracts were combined and the acetone was removed by vacuum distillation.

In the second method, the liver (580 g.) was homogenized with 1 l. of 0·6 N-$HClO_4$. On standing overnight the liver tissue, owing to its high content of oil, rose to the surface and most of the extract could be removed by aspiration. Extraction with a second 1 l. of the acid was performed in the same way. The combined extracts were neutralized with 5 N-KOH and filtered.

Extracts obtained by either of these methods were treated as follows. Excess of basic lead acetate was added and the white precipitate allowed to settle for several hours. The clear supernatant was then poured off and the precipitate collected by centrifuging. After three water washes in the centrifuge, the precipitate was suspended in 300 ml. of water and decomposed with excess of $H_2S$. The PbS was removed by filtration and the clear filtrate concentrated *in vacuo* to about 50 ml. After deionizing with Dowex 2 and Dowex 50 the filtrate was tested for *scyllo*- and *myo*-inositol.

### *Free inositols of* Raia clavata *liver*

The aqueous extract prepared as described in the section above was treated in the same way as the acorn extract to induce crystallization. An extract prepared from 750 g. of liver gave 230 mg. of a white crystalline solid. A sharp melting point could not be obtained, but the gradual decomposition between 250° and 300° suggested that *scyllo*inositol was the main component. For confirmation the product was acetylated.

*Acetylation.* The crystals (222 mg.) were dried at 98° *in vacuo* over $P_2O_5$ for 1 hr. They were then warmed with 10 ml. of acetic anhydride containing 0·3% (v/v) of 72% (w/w) $HClO_4$ until all had dissolved. After 20 min. the mixture was poured into 500 ml. of ice–water. An oil was produced, which solidified after 2 hr. in the cold with occasional stirring. The solid was filtered off at the pump, washed well with cold water and dried over $P_2O_5$ *in vacuo* at room temperature for 18 hr. On treatment with boiling absolute ethanol, a little of the product dissolved, leaving 265 mg. of white needles, m.p. (uncorr.) 285–286°. Hann & Sando (1926) give the melting point of *scyllo*inositol hexaacetate as 291° when recrystallized from acetic acid.

*Ionophoresis.* A solution obtained from a second sample of liver gave two spots on electrophoresis in borate solution. The stronger one corresponded with *scyllo*inositol and the weaker (roughly one-fifth the amount) with *myo*inositol. Since no attempt at recrystallization had been made, which would concentrate the less soluble *scyllo*inositol, these relative amounts apply to the free inositol isomers in the original liver tissue. The same is true of the acorn electrophoresis results.

### *Preparation of acorn phospholipids*

The residue from the extraction of the acorns (880 g.) with acetone (2 × 1·2 l.) was extracted twice with 1·5 l. of chloroform–methanol (2:1, v/v). The combined chloroform–methanol extracts were concentrated to a volume of about 30 ml. *in vacuo*. Acetone (500 ml.) was then added to precipitate crude phospholipid and remove pigment. After 2 days at 4°, the precipitate was separated by centrifuging and shaken well with 50 ml. of chloroform. After filtering to remove non-lipid material, the chloroform solution was concentrated *in vacuo* to 5 ml. and treated with 100 ml. of acetone. In one case no precipitate could be obtained until a few drops of aqueous 20% $CaCl_2$ solution were added. The phospholipid precipitate was spun down and stored in chloroform solution. The average yield of phospholipid (as phosphorus) was 2 mg./kg. of fresh acorns.

### *Hydrolysis of acorn phospholipids*

From 880 g. of acorns a chloroform solution containing only about 2 mg. of phospholipid phosphorus was finally obtained. A sample equivalent to 740 $\mu$g. of phosphorus was dried and hydrolysed with 0·4 ml. of 2 N-HCl in a sealed tube at 110° overnight. The cooled hydrolysate was treated with charcoal (previously shown not to absorb the inositols) and then with Dowex 2 ($OH^-$ form). The hydrolysate was evaporated to a small volume and ionophoresis carried out on samples corresponding to about 200 $\mu$g. of inorganic phosphorus in the original hydrolysate. A strong spot in the *myo*inositol position was seen, but no *scyllo*-inositol.

*Washing procedure.* Since phospholipids are noted for their ability to carry non-lipid contaminants into solution in organic solvents, a second sample of the chloroform solution of acorn phospholipids was washed before hydrolysis.

A 3 ml. portion of the chloroform solution (360 $\mu$g. of phosphorus) was shaken well with 1 ml. of 0·1 M-KCl and 0·5 ml. of 2 N-HCl. The mixture was centrifuged and the upper aqueous layer removed. To the lower layer and interfacial material 0·5 ml. of 2 N-HCl, 1 ml. of 0·1 M-KCl and 0·5 ml. of methanol were added. The mixture was again shaken and centrifuged. The aqueous layer was removed. The interfacial lipid and lower chloroform layer were hydrolysed as described above. Again, electrophoresis showed only *myo*inositol, in amounts that showed that very little could have been removed by the washing. The *myo*-inositol was therefore a lipid component.

### *Preparation of* Raia clavata *liver phospholipid*

The fish liver (500 g.) was homogenized with 1 l. of acetone. The solvent, which extracted much oil from the tissue, was filtered off at the pump and the residue re-extracted with 1 l. of acetone. After this second portion of acetone had been filtered, the solid was extracted twice with 1 l. of chloroform–methanol (2:1, v/v). These two

extracts were combined and evaporated *in vacuo* to a volume of about 30 ml. Excess of acetone was then added and the mixture kept at 4° overnight. The precipitate was centrifuged down and suspended in 30 ml. of chloroform. Insoluble matter was removed by centrifuging and 200 ml. of absolute ethanol added to the lipid solution, precipitating a 'kephalin' fraction. The supernatant was concentrated *in vacuo* to approximately 10 ml. and a 'lecithin' fraction precipitated with 200 ml. of acetone. This lecithin was stored in chloroform solution. The kephalin fraction was redissolved in 10 ml. of chloroform, precipitated once more with 200 ml. of absolute ethanol and also stored in chloroform solution. The approximate yield of phospholipid (as phosphorus)/kg. of fresh liver was lecithin 10 mg., kephalin 6 mg.

*Hydrolysis of* Raia clavata *liver phospholipids*

Samples of the lecithin and kephalin fractions were washed and hydrolysed as described for the acorn lipid. Chromatography of the hydrolysate in propan-2-ol–acetic acid–water (3:1:1, by vol.) showed glycerol in both hydrolysates. Choline was seen in the lecithin hydrolysate. Three ninhydrin-reactive spots, the strongest of which resembled serine in position, were seen in the kephalin hydrolysate.

After treatment with charcoal and Dowex 2, samples of the hydrolysate were subjected to borate ionophoresis. A strong *myo*inositol spot was given by the kephalin hydrolysate, a weak one by the lecithin. No *scyllo*inositol could be detected.

## DISCUSSION

The present work shows that the amount of free *myo*inositol in the acorn and in ray liver is small compared with that of free *scyllo*inositol. Müller (1912) failed to find any of the *myo*-compound in the tissues of the spur dogfish, though *scyllo*inositol was isolated without difficulty.

In spite of the predominance of *scyllo*inositol in the tissues studied, only the *myo*-isomer is selected for phospholipid synthesis. It is difficult to exclude completely the presence of 'phosphoscyllitides', but the evidence suggests that they cannot be present in more than minute amounts.

The chemical structures of *myo*inositol (I) and *scyllo*inositol (II) differ very little. The only difference lies in the presence of an axial 2-hydroxyl in *myo*inositol, giving (if the ring is regarded as planar) a trio of *cis*-hydroxyl groups instead of the all-*trans* *scyllo*inositol structure. Since *scyllo*inositol is not incorporated into the lipids it would appear that the enzyme incorporating inositol into the phospholipid is specific for the *myo*-structure. If the pathway (Paulus & Kennedy, 1960) applies to the tissues studied here, this enzyme catalyses the reaction:

CDP-diglyceride + *myo*inositol → CMP + phosphatidylinositol.

The actual linkage of *myo*inositol to phosphate in phosphatidylinositol, the only phosphoinositide completely characterized so far, is through the 1-hydroxyl (Pizer & Ballou, 1959), but it is likely that the 2-hydroxyl and other neighbouring groups affect the enzyme–substrate interaction. Paulus & Kennedy showed that D-inositol (III), L-inositol and pinitol (5-*O*-methyl-D-inositol) failed to react. Taking these compounds and *scyllo*inositol into consideration, the minimum requirements for enzymic reaction appear to be a trio of adjacent *cis*-hydroxyl groups (i.e. equatorial hydroxyl group on C-1 and C-3, axial on C-2).

## SUMMARY

1. Free *scyllo*inositol and *myo*inositol have been found in the liver of the common ray (*Raia clavata*) and in the acorn, the latter compound in relatively small amounts.

2. In the phospholipids of these tissues *myo*-inositol, but no *scyllo*inositol, could be detected.

I should like to thank Professor A. C. Frazer for his interest in the work.

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# Indirect Analysis of Corticosteroids

## 5. THE DETERMINATION OF 17-DEOXYCORTICOSTEROIDS

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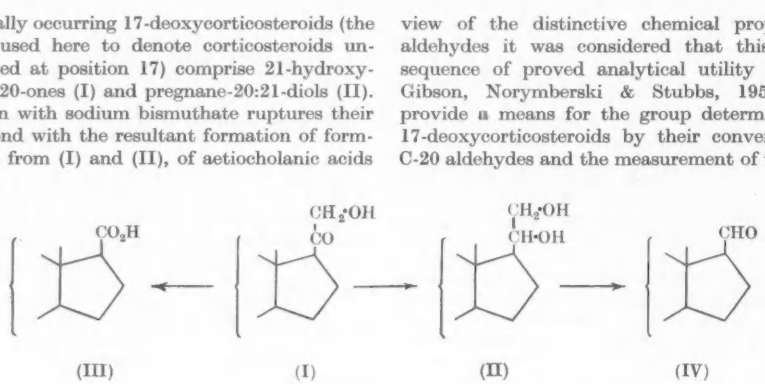
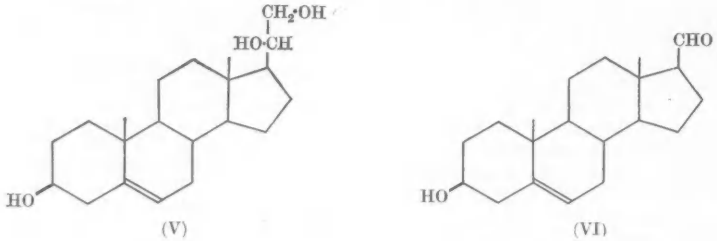
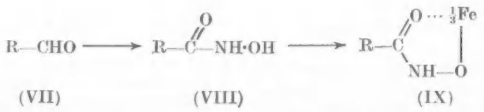
Naturally occurring 17-deoxycorticosteroids (the term is used here to denote corticosteroids unsubstituted at position 17) comprise 21-hydroxypregnan-20-ones (I) and pregnane-20:21-diols (II). Oxidation with sodium bismuthate ruptures their 20–21 bond with the resultant formation of formaldehyde from (I) and (II), of aetiocholanic acids (III) from (I) and of 21-norpregnan-20-als (IV) from (II) (Brooks & Norymberski, 1953). The last conversion was presumed; it is now established by the isolation of 3$\beta$-hydroxy-21-norpregn-5-en-20-al (VI) (as its acetate) from the reaction of pregn-5-ene-3$\beta$:20$\beta$:21-triol (V) with sodium bismuthate. Reduction with borohydride followed by oxidation with sodium bismuthate was expected to convert 20:21-ketols (I) into C-20 aldehydes (IV) as well. In view of the distinctive chemical properties of aldehydes it was considered that this reaction sequence of proved analytical utility (Appleby, Gibson, Norymberski & Stubbs, 1955) might provide a means for the group determination of 17-deoxycorticosteroids by their conversion into C-20 aldehydes and the measurement of the latter.




The Angeli–Rimini reaction (Angeli, 1892; Rimini, 1901) was chosen for the detection of aldehydes because of its specificity: only aldehydes are known to react and only those which lack substituents interacting with the aldehyde group. The reaction is usually performed according to Rimini by treating aldehydes (VII) with benzenesulphohydroxamic acid in a weakly alkaline solution. The hydroxamic acids (VIII) formed are then detected in the reaction mixture by conversion into their purple ferric complexes (IX).

*Part 4: Norymberski & Stubbs (1956).

*Preliminary experiments*

The conditions outlined above were found unsuited to the accurate determination of small amounts ($< 1\,\mu$mole) of aldehydes because of large and variable reagent blanks presumably due to surplus benzenesulphohydroxamic acid and to the formed benzenesulphinic acid (Struck, 1956). However, when the formed hydroxamic acid was extracted with ethyl acetate, the interfering substances remained in the aqueous phase. The ferric hydroxamate was formed by shaking the extract with a solution of ferric nitrate in perchloric acid. The complex was then found in the organic phase but was quantitatively transferred into the aqueous phase on addition of light petroleum; by suitably adjusting the volumes of extract, reagent and diluent almost a five-fold concentration of the complex was achieved. Its spectrum in either phase showed the typical absorption maximum at 510 m$\mu$ (Fig. 1). Either procedure was found satisfactory for the determination of pure aldehydes but only the latter, involving the preparation of an aqueous concentrate, was applicable to the intended determination of 17-deoxycorticosteroids in the urine. Therefore, what follows refers to results obtained by the latter technique, with 3-oxobisnorchol-4-en-22-al as compound of reference. In terms of this compound (= 100), 3$\beta$-acetoxynorpregn-5-en-20-al (VI-acetate) was found to have a colour equivalent of 92 (mole/mole). Colorimetric measurements were found to obey Beer's law (Fig. 2, curve *A*). Their reproducibility was improved by the use of Allen's correction (Allen, 1950). Agreement between duplicate determinations carried out concurrently was good (coefficient of variation 3%, calculated from 26 measurements); agreement between determinations carried out on different days was less satisfactory (coefficient of variation 8%, calculated from 32 measurements). It is therefore desirable that all determinations be expressed in terms of a model compound assayed concurrently with the analytical sample under investigation.

Three 20:21-ketols and one 20:21-glycol (compounds 1, 2, 3 and 6 in Table 1) were treated consecutively with potassium borohydride and sodium bismuthate: by the analytical test outlined, all four compounds gave aldehydes in high yield (Table 1, column *A*). The formed ferric hydroxamates exhibited absorption spectra identical with those derived from pure aldehydes. Submitted to the same sequence of reactions, aldosterone and cholestane-3$\beta$:5$\alpha$:6$\beta$-triol failed to yield ferric hydroxamates, presumably owing to interaction between the formed aldehyde group and a neighbouring oxygen function. Four compounds of the androstane series and one of the oestrane series with vicinal oxygen functions at positions 16 and 17 gave ferric hydroxamates in poor yield (Table 1), interaction presumably occurring between the two aldehyde groups of the expected 16:17-*seco*dialdehydes.

The proposed determination of 17-deoxycorticosteroids in the urine was facilitated by their stability towards hot mineral acid (Reichstein & Shoppee, 1943; Tompsett, 1953; see also Table 1, column *B*) since it permitted the determination to be performed on the readily prepared extracts of acid-treated urines. Preliminary experiments showed that such extracts, when analysed for 17-deoxycorticosteroids, gave rise to asymmetrical absorption spectra, indicating the presence of material absorbing non-specifically in the spectral region 400–600 m$\mu$. In order to ascertain the spectroscopic properties of this material, determinations were carried out on 'urinary blanks' obtained by omitting the addition of ferric ion at the final stage of the assay. The blanks exhibited approximately linear absorption

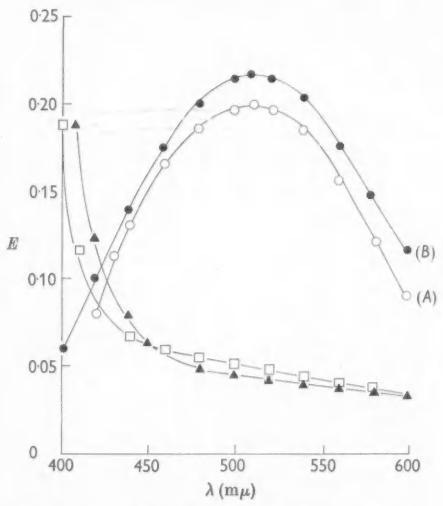



Fig. 1. Spectra of the ferric hydroxamate from 3-oxobisnorchol-4-en-20-al: ●, 0·5 $\mu$mole in ethyl acetate extract; ○, 0·1 $\mu$mole in aqueous concentrate; ▲, reagent blank in ethyl acetate; □, reagent blank in aqueous concentrate. ● and ○ have been corrected by subtraction of corresponding reagent blanks.

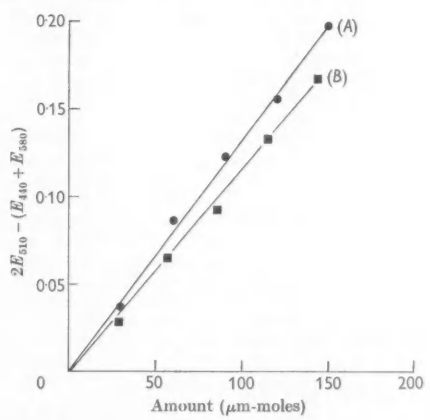



Fig. 2. Proportionality in the determination of aldehydes and of 17-deoxycorticosteroids. ●, 3-Oxobisnorchol-4-en-22-al; ■, 3$\alpha$:21-dihydroxy-5$\beta$-pregnane-11:20-dione, added to urine.

Table 1. *Determination of ketols and glycols*

*A*: Direct determination; *B*: determination after treatment with hot HCl; *C*: compound added to urine and determined by standard procedure. Results are in terms of 3-oxobisnorchol-4-en-22-al (mole/mole); mean values are given, with their S.D.; figures in parentheses denote the number of determinations.

| | Compound | Procedure *A* (% found) | Procedure *B* (% found) | Procedure *C* (% found) | Quantity assayed (μg.) |
|---|---|---|---|---|---|
| 1 | 11-Deoxycorticosterone | 94±2 (25) | 87±2 (3) | 82±2 (2) | 20–250 |
| 2 | Corticosterone | 93±7 (8) | 86±4 (8) | 103±6 (5) | 20–50 |
| 3 | 3α:21-Dihydroxy-5β-pregnane-11:20-dione | 99±2 (7) | — | 96±7 (12) | 10–100 |
| 4 | 3α:21-Diacetoxy-5β-pregnane-11:20-dione | — | — | 105±4 (3) | 50 |
| 5 | 5β-Pregnane-3α:11β:20β:21-tetrol | — | 82±2 (2) | 83±2 (2) | 40–45 |
| 6 | Pregn-5-ene-3β:20β:21-triol | 90±3 (2) | — | 87±2 (4) | 50 |
| 7 | Aldosterone | 0 (1) | — | — | 12·5 |
| 8 | 3α:17α:21-Trihydroxy-5β-pregnane-11:20-dione | 0·6±0 (3) | — | 0±0 (3) | 360–500 |
| 9 | Cholestane-3β:5α:6β-triol | 0±0 (2) | — | — | 22 |
| 10 | 3β:16α-Dihydroxyandrost-5-en-17-one | 18±0 (2) | — | — | 40 |
| 11 | 3β:17β-Dihydroxyandrost-5-en-16-one | 23±3 (4) | — | — | 40–170 |
| 12 | Androst-5-ene-3β:16α:17β-triol | 19±0 (2) | — | — | 41 |
| 13 | Androst-5-ene-3β:16β:17β-triol | 18±0 (2) | — | — | 40 |
| 14 | Oestriol methyl ether | 23±0 (2) | — | — | 25 |

whilst analytical samples treated in the usual manner, when corrected by subtraction of the blanks, gave the typical spectra of ferric hydroxamates (Fig. 3). It follows that the determination of 'urinary blanks' is unnecessary since their colorimetric contribution can be eliminated by the application of Allen's correction. Parallel determinations of samples of a urine treated with acid and with β-glucuronidase respectively gave practically identical results (see Table 2); hence, there is no reason to suspect that treatment with acid leads to the formation of artifacts interfering with the assay. The standard procedure was found accurate (coefficient of variation between duplicate determinations 4%, calculated from 67 measurements), it accounted satisfactorily for varying amounts of pure 17-deoxycorticosteroids added to the urine (Table 1, column *C*; Fig. 2, curve *B*), and its results were not affected by the presence of relatively large quantities of 3α:17:21-trihydroxy-5β-pregnane-11:20-dione (tetrahydrocortisone) (Table 1). Average excretion of 17-deoxycorticosteroids by 16 healthy men was 3·4 mg./24 hr. (range 2·3–5·2; S.D. 0·8) and by eight healthy women it was 3·1 mg./24 hr. (range 2·0–4·7; S.D. 1·0).

Since oxidation with sodium bismuthate of 21:21-ketols (I) yields aetiocholanic acids (III) and that of 20:21-glycols (II) C-20 aldehydes (IV), it is clear that omission of the reduction step from the standard procedure provides a method for the determination of the latter subgroup (II) of 17-deoxycorticosteroids. 20:21-Glycols were so determined in urines from 12 normal subjects and were found to account on the average for 71% (range 53–80; S.D. 8) of the total 17-deoxycorticosteroids (see Table 3).

Brief reference to this work was made previously (Norymberski, 1961).

## MATERIALS AND METHODS

### *Preparation of 3β-acetoxy-21-norpregn-5-en-20-al (VI-acetate)*

Pregn-5-ene-3β:20β:21-triol (V) (100 mg.) in aqueous acetic acid (100 ml.; 50%, v/v) was shaken for 5 min. with sodium bismuthate (10 g.). Surplus reagent was filtered off, washed with a little methanol, the filtrate was diluted with water (100 ml.) and extracted with ethyl acetate. The

Table 2. *Comparison of two hydrolytic procedures in the assay of urinary 17-deoxycorticosteroids*

Subjects 1–3 were untreated obese women. Subject 4 was an obese man treated with corticotrophin.

| Subject no. | 17-Deoxycorticosteroids (mg./24 hr.) Hydrolysis by acid | Hydrolysis by enzyme |
|---|---|---|
| 1 | 4·5 | 4·0 |
| 2 | 4·7 | 4·55 |
| 3 | 4·4 | 3·9 |
| 4 | 11·5 | 12·5 |

Table 3. *20:21-Glycols and 20:21-ketols in normal human urine*

For details see text.

| Subject no. | 17-Deoxy-corticosteroids (mg./24 hr.) | 20:21-Glycols (mg./24 hr.) | 20:21-Ketols (mg./24 hr.) |
|---|---|---|---|
| 1 | 2·9 | 2·1 | 0·8 |
| 2 | 4·1 | 3·2 | 0·9 |
| 3 | 3·2 | 2·5 | 0·7 |
| 4 | 3·4 | 2·4 | 1·0 |
| 5 | 2·5 | 1·8 | 0·7 |
| 6 | 5·2 | 3·6 | 1·6 |
| 7 | 2·7 | 1·9 | 0·8 |
| 8 | 3·3 | 2·6 | 0·7 |
| 9 | 1·9 | 1·4 | 0·5 |
| 10 | 4·7 | 2·8 | 1·9 |
| 11 | 3·6 | 2·8 | 0·8 |
| 12 | 4·4 | 2·3 | 2·1 |
| Mean | 3·5 | 2·5 | 1·0 |
| S.D. | 1·0 | 0·6 | 0·5 |

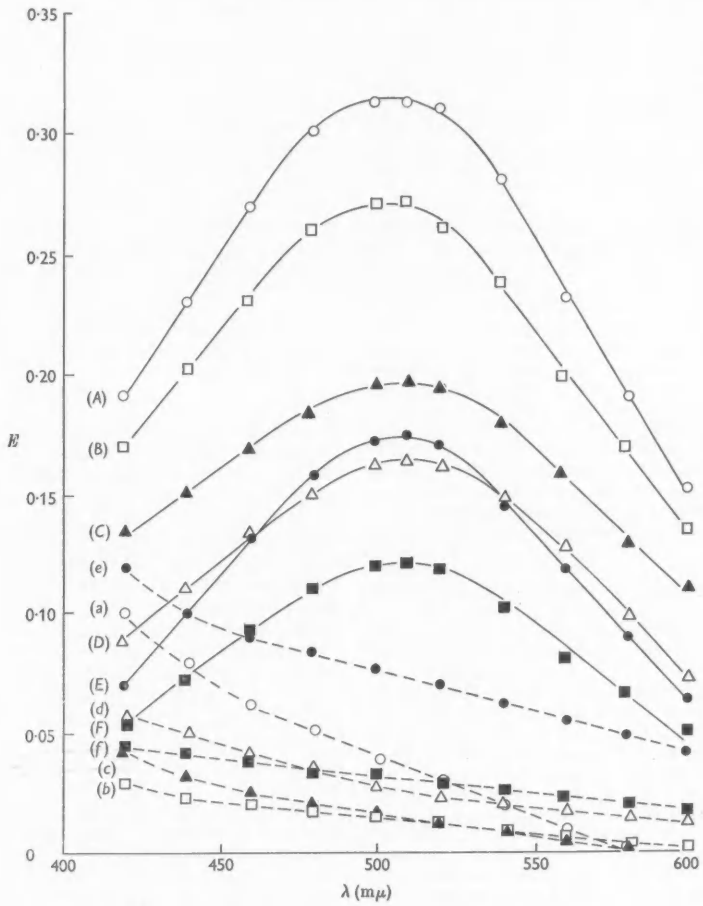



Fig. 3. Spectra obtained in the assay of six normal urines (curves *A–F*) and the corresponding 'urinary blanks' (curves *a–f*). All readings are corrected as described in the text.

crude product was treated with acetic anhydride in pyridine overnight at room temperature. The pale-yellow product was decolorized with charcoal in hexane, whereby colourless crystals of (VI)-acetate (95 mg.) were obtained; m.p. 125–140° (Kofler stage). Material recrystallized from hexane had m.p. 130–140° (Kofler stage), 145–152° (capillary), 156–157° (evacuated capillary); $[\alpha]_D -20 \pm 2°$ in $CHCl_3$ (*c*, 1·57); $\nu_{max.}$ in $CS_2$ for CHO 2700 and 1720 (shoulder) cm.$^{-1}$, for $CH_2 \cdot CO_2$ 1730 and 1240 cm.$^{-1}$ (Found: C, 76·2; H, 9·6. Calc. for $C_{22}H_{32}O_3$: C, 76·7; H, 9·4%). Miescher, Hunziger & Wettstein (1940) reported m.p. 169–171° $[\alpha]_D -13·5 \pm 4°$.

*Analytical*

All solvents but methylene dichloride and ethylene dichloride were of analytical grade; all but light petroleum (b.p. 60–80°) were distilled. Colorimetric measurements were carried out with a Hilger Uvispek spectrophotometer in microcells of 1 cm. light-path and 0·2 ml. capacity.

(i) *Determination of aldehydes.* The analytical sample, containing 5–50 μg. of aldehyde, is dissolved in *tert.*-butanol (0·2 ml.), treated with a freshly prepared solution of benzenesulphohydroxamic acid in *tert.*-butanol (0·1 ml.; 0·1 M) and with a solution (0·1 ml.) of sodium hydroxide (0·125 N) in aq. *tert.*-butanol (50%, v/v). After 30 min. at

room temperature, water (0·5 ml.) is added and after a further 15 min. the mixture is shaken with ethyl acetate (1·0 ml.) for 15 min. and then centrifuged at 4000 rev./min. for 5 min. A portion of the top layer (1·0 ml.) is carefully removed and shaken for 5 min. with a solution (0·1 ml.) of ferric nitrate (0·5 N) in perchloric acid (2·5 N); light petroleum (2·0 ml.; b.p. 60–80°) is added and the mixture shaken again. It is then centrifuged, the organic phase carefully removed and the aqueous phase washed with a mixture of ethyl acetate and light petroleum (3·0 ml.; 1:2, v/v). The extinction of the aqueous layer is determined at 440, 510 and 580 mμ against a process blank obtained by submitting *tert.*-butanol (0·2 ml.) alone to the treatment. The readings are evaluated according to Allen (1950) and expressed in terms of a pure standard aldehyde: here, 3-oxobisnorchol-4-en-22-al.

Pure aldehydes and those prepared from pure 17-deoxycorticosteroids (see below) can be determined in the ethyl acetate extract after its treatment with the ferric reagent. When a blank is required, accounting for coloured impurities ('urinary blank' with processed urine extracts), the procedure is varied as follows. The volumes of solvents and solutions employed are doubled. From the ethyl acetate extract two equal samples (1·0 ml. each) are taken; one sample (*A*) is treated with the ferric reagent, the other (*B*) with perchloric acid only. All following operations are performed as described above. Preparation of the process blank is modified in the same manner (samples *a* and *b*). The extinction of the ferric hydroxamate is found as $(A - B) - (a - b)$.

(ii) *Determination of 17-deoxycorticosteroids.* The analytical sample, containing 10–100 μg. of 17-deoxycorticosteroids, is dissolved in *tert.*-butanol (0·2 ml.) and treated with a freshly prepared aqueous solution of potassium borohydride (0·1 ml.; 20%, w/v) overnight at room temperature. Aqueous acetic acid (3 ml.; 40%, v/v) and sodium bismuthate (0·5 g.) are added, the mixture shaken for 10 min., centrifuged, a portion of the supernatant (2·5 ml.) treated with aq. sodium metabisulphite (0·05 ml.; 20%, w/v) and with hydrochloric acid (1 ml.; 5 N) and then shaken with methylene dichloride (3·0 ml.) for 15 min. The aqueous phase is removed and the extract (i.e. lower phase) washed successively with water (1 ml.), 3 N-NaOH (1 ml.; shaken for 5 min.) and water (2 × 1 ml.). The washed extract is filtered through anhydrous sodium sulphate and a portion (2·0 ml.) evaporated to dryness, care being taken that the residue is not overheated. The residue is treated as described under (i).

Selective determination of 20:21-glycols is carried out by omitting treatment with potassium borohydride but otherwise in exactly the same manner as above.

The time of oxidation with sodium bismuthate was varied between 5 and 15 min. with satisfactory results. Shorter and longer reaction times lead to lower yields of aldehydes.

(iii) *Determination of 17-deoxycorticosteroids in the urine.* (*a*) With acid hydrolysis (standard procedure). A 24 hr. urine specimen is made up to 2·0 l. with water. A sample (60·0 ml.) of the diluted urine with conc. hydrochloric acid (20·0 ml.) in a glass-stoppered tube (approx. capacity 120 ml.) is heated for 10 min. in a boiling-water bath and then rapidly cooled. The mixture is shaken with ethylene dichloride (20·0 ml.) for 15 min., most of the aqueous phase removed with suction and the extract transferred into a glass-stoppered centrifuge tube (approx. capacity 40 ml.). Any emulsion is broken down by centrifuging, the remaining aqueous phase removed and the extract washed successively with water (4 ml.), 3 N-NaOH (4 ml.; shaken for 5 min.) and water (2 × 4 ml.). The washed extract is filtered through a layer of anhydrous sodium sulphate and a portion (15·0 ml.) evaporated to dryness. The residue is treated as under (ii).

(*b*) With enzymic hydrolysis. One urine specimen was treated with bacterial β-glucuronidase (Sigma Chemical Co.) (300 Sigma units/ml., pH 6·8, 36 hr. at 37°), three other specimens with bovine β-glucuronidase (Ketodase, Warner–Chilcott) (450 Fishman units/ml., pH 4·8, 60 hr. at 37°; see Talalay, Fishman & Huggins, 1946). Each urine was then extracted with chloroform (3 × 0·5 vol.), the extract washed successively with 3 N-NaOH (0·2 vol.) and water (3 × 0·2 vol.), dried with anhydrous sodium sulphate and evaporated to dryness *in vacuo*. Suitable portions of this material were assayed as described under (ii) and the results compared with those obtained by method (*a*) (see Table 2).

## DISCUSSION

An earlier attempt at the group determination of urinary 17-deoxycorticosteroids (Tompsett, 1953), as acid-resistant formaldehydogenic substances, proved unsuccessful when it was shown that cortisone and Reichstein's substance S (and presumably also other 17:21-diol-20-ones) significantly contribute to the assay (Marrian, Paterson & Atherden, 1953). The α-ketolic subgroup of 17-deoxycorticosteroids (20:21-ketols) has been determined as reducing substances non-chromogenic in the Porter–Silber reaction (Romani, Bugard & Fischer, 1956) and as reducing substances present in a chromatographic zone considered free of other reducing material (Richardson *et al.* 1955). Currently, the analytical method of choice is the determination of individual compounds of the group after their chromatographic separation. So far, 12 such compounds (i–xii, Table 4) have been found in human urine. They were originally isolated from the urine of subjects treated with corticotrophin, corticosterone, 11-dehydrocorticosterone or 11-deoxycorticosterone (Table 4); seven of them (i–vii), all 20:21-ketols, were later found also in the urine of untreated normal subjects (Romani, 1956*a*, *b*; Romani & Ableaux-Fernet, 1956).

The present method is considered specifically to determine neutral acid-resistant α-glycols and α-ketols which on consecutive treatment with borohydride and with sodium bismuthate yield non-volatile aldehydes. Among steroids known to occur in the human urine these requirements are met by 17-deoxycorticosteroids and by the 16:17-glycols of the androstane series. Since the latter group of compounds is excreted by man in relatively small amounts (Fotherby, Colas, Atherden & Marrian, 1957) and since they are converted into ferric hydroxamates in very poor yield (see Table 1) it is considered that their contribution to the assay

Table 4. *17-Deoxycorticosteroids in human urine*

| | Compound | Reference |
|---|---|---|
| (i) | 21-Hydroxypregn-4-ene-3:20-dione (11-Deoxycorticosterone) | Richardson *et al.* (1955) |
| (ii) | 3α:21-Dihydroxy-5β-pregnan-20-one | Richardson *et al.* (1955) |
| (iii) | 21-Hydroxypregn-4-ene-3:11:20-trione (11-Dehydrocorticosterone, substance A) | Touchstone, Bulaschenko & Dohan (1955) |
| (iv) | 11β:21-Dihydroxypregn-4-ene-3:20-dione (Corticosterone, substance B) | Touchstone, Bulaschenko, Richardson & Dohan (1954) |
| (v) | 3α:11β:21-Trihydroxy-5β-pregnan-20-one | Touchstone *et al.* (1954) |
| (vi) | 3α:11β:21-Trihydroxy-5α-pregnan-20-one | Touchstone *et al.* (1954); Engel, Carter & Springer (1954) |
| (vii) | 3α:-21-Dihydroxy-5β-pregnane-11:20-dione | Touchstone *et al.* (1954); Engel *et al.* (1954) |
| (viii) | 3α:-21-Dihydroxy-5α-pregnane-11:20-dione | Peterson (1959) |
| (ix) | 3β:21-Dihydroxypregn-5-en-20-one | Dobriner & Lieberman (1952) |
| (x) | 11β:20β:21-Trihydroxypregn-4-en-3-one | Richardson, Bulaschenko & Dohan (1958) |
| (xi) | 11β:20α:21-Trihydroxypregn-4-en-3-one | Bulaschenko, Richardson & Dohan (1960) |
| (xii) | 20β:21-Dihydroxypregn-4-ene-11:20-dione | Bulaschenko *et al.* (1960) |

is negligible. The 16:17-ketols being unstable to acid (Marrian, Watson & Panattoni, 1957) need not be here considered. The excellent correlation found between the excretion of 17-hydroxycorticosteroids and of 17-deoxycorticosteroids by normal untreated subjects and by subjects whose adrenal function was suppressed by treatment with 9α-fluorocortisol (Norymberski, 1961) indicates adrenal origin of the compounds determined by the present method. Assuming that the assay is specific, its elaboration to the separate determination of 20:21-glycols and 20:21-ketols reveals the presence of considerable quantities of the former in the urine of healthy men and women. Although three 20:21-glycols containing the 4-en-3-one system (x–xii) were found in small quantities in the urines of patients treated with corticosterone or with the acetate of 11-dehydrocorticosterone (Richardson *et al.* 1958; Bulaschenko, Richardston & Dohan, 1960) it is most unlikely that these compounds account for more than a small fraction of the 20:21-glycols detected by the group assay in the urine of normal subjects. It is therefore suggested that man excretes substantial amounts of as yet unidentified 20:21-glycols.

## SUMMARY

1. The Angeli–Rimini reaction was adapted to the determination of small quantities ($\geqslant 10\,\mu$m-moles) of aldehydes.

2. A method was developed for the group determination of 17-deoxycorticosteroids by their conversion into 21-norpregnan-20-als and the measurement of the latter by means of the modified Angeli–Rimini reaction. The method was successfully applied to the determination of 17-deoxycorticosteroids in extracts of acid-treated urines.

3. The above method was modified to permit the separate determination of 21-hydroxypregnan-20-ones and of pregnane-20:21-diols. Applied to urines of normal subjects, the differential assay revealed the presence of substantial quantities of as yet unidentified compounds of the 20:21-diol group.

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# The Intracellular Distribution, Latency and Electrophoretic Mobility of L-Glutamate–Oxaloacetate Transaminase from Rat Liver

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Previous studies of the distribution of L-glutamate–oxaloacetate transaminase in subcellular fractions, obtained by differential centrifuging of liver homogenates, have all shown that this enzyme is present in both supernatant and mitochondria, with a somewhat higher percentage of total activity in the supernatant fraction (Müller & Leuthardt, 1950; Asada, 1958; Gaull & Villee, 1960). There are no previous reports of investigations of the latency and activation of L-glutamate–oxaloacetate transaminase in mitochondria, although an increase of activity in liver homogenates during storage has been reported (Schmidt, Schmidt & Wildhirt, 1958) and aging is known to activate latent mitochondrial enzymes. Green, Leloir & Nocito (1945) reported that purified L-glutamate–oxaloacetate transaminase could be separated by electrophoresis into three components and that activity was associated with two of these. It has recently been shown (Fleisher, Potter, Wakim, Pankow & Osborne, 1960) that two L-glutamate–oxaloacetate transaminases with different electrophoretic mobilities have different substrate affinities and pH-dependence. The following experiments show a relationship between the electrophoretic mobility of the enzyme and its localization within the cell.

## EXPERIMENTAL

### *Enzyme-assay procedures*

Several procedures were used to assay L-glutamate–oxaloacetate transaminase. Units of transaminase activity were defined as μmoles of oxaloacetate or L-glutamate formed/hr. at 25°.

*L-Glutamate–oxaloacetate-transaminase assay by the method of Reitman & Frankel* (1957). The amount of oxaloacetate formed during incubation for 1 hr. at 37° was measured colorimetrically with 2:4-dinitrophenylhydrazine. The results were converted into μmoles of oxaloacetate formed/hr. at 25° (King, 1960).

*L-Glutamate–oxaloacetate-transaminase assay by the method of Karmen* (1955). In this method the rate of formation of oxaloacetate is determined, reduced diphosphopyridine nucleotide (DPNH) and malic dehydrogenase being added to the assay system so that DPNH is oxidized as oxaloacetate is produced. The resulting decrease in extinction at 340 mμ was followed in a Unicam SP. 500 spectrophotometer; the progress curve was linear. A fall in $E$ of 0·01 is equivalent to the formation of 4·83 μm-moles of oxaloacetate in the reaction mixture. The temperature was maintained at 25° by use of a cell housing through which water from a thermostat was circulated. A higher concentration of L-aspartate, 66·6 mM, was used than that in the original method (33·3 mM). The concentration of α-oxoglutarate was 6·7 mM.

It was found that in the presence of $NH_4^+$ ions some glutamic-dehydrogenase activity is measured in the Karmen method. The commercial malic dehydrogenase added in this test contains $(NH_4)_2SO_4$, 9 mM final concentration. To correct for this the oxidation of DPNH was followed in the presence of all reactants except L-aspartate and this control rate was subtracted from the final rate after adding aspartate, allowance being made for dilution. A correction of up to 10% was sometimes necessary. A slight inhibition of transamination, 2–5%, due to these $NH_4^+$ ions, was ignored.

*L-Glutamate–oxaloacetate-transaminase assay by the method of Cammarata & Cohen* (1951). The rate of oxaloacetate production is measured spectrophotometrically at 280 mμ. Preliminary experiments showed that the substrate concentrations in the original descriptions by Cammarata & Cohen (1951) were suboptimum for mitochondrial preparations; hence they were both increased from the

Table 1. *Activation of glutamate–oxaloacetate transaminase in rat-liver homogenates*

The values are means of three or four different experiments. Units are μmoles of DPNH oxidized or oxaloacetate produced/hr./mg. of liver (wet wt.) at 25°. Tramsaminase was assayed by the method of Karmen (1955). For experimental details see text.

| | Water homogenate | 0·25 M-Sucrose homogenate | | |
|---|---|---|---|---|
| | | Untreated | Waring Blendor, 4 min. | Ultrasonic disintegrator, 1 min. |
| Glutamic dehydrogenase | 2·32 | 0·71 | 2·88 | 2·35 |
| L-Glutamate–oxaloacetate transaminase | 5·30 | 1·55 | 5·75 | 5·43 |

20 mM of the original method, L-aspartate to 133 mM and α-oxoglutarate to 50 mM. Preincubation with pyridoxal phosphate was omitted because only crude enzyme preparations were being assayed. 0·1 M-Potassium phosphate buffer, pH 7·5, was used, as in the other methods, instead of 0·05 M-phosphate buffer, pH 7·4. It was found necessary in this assay to include blanks when testing mitochondria to allow for decrease in *E*, due presumably to swelling of these particles during the assay. The routine blank adopted contained mitochondrial preparation suspended in the phosphate buffer.

*L-Glutamate–oxaloacetate-transaminase assay by the method of Müller & Leuthardt* (1950). The glutamic acid formed in a given time is measured by the glutamic-decarboxylase method of Krebs (1948). This method was modified by the use of a higher concentration of Cetavlon, as advised by Meister, Sober & Tice (1951).

A further modification was the use of higher substrate concentrations, namely 100 mM-L-aspartate and 50 mM-α-oxoglutarate. Incubation was for 30 min. at 25°. The reaction was stopped by immersion of the reaction mixture for 5 min. in a boiling-water bath. This method gave results linear with respect to enzyme concentration only up to approx. 1·5 μmoles of glutamic acid/hr. An unincubated enzyme–substrate mixture was used as the routine blank.

*Glutamic-dehydrogenase assay.* Glutamic dehydrogenase was estimated spectrophotometrically with the following reaction mixture: 87 μM-DPNH; 73 mM-potassium phosphate buffer, pH 7·5; 0·167 M-$(NH_4)_2SO_4$; 0·2 ml. of enzyme preparation. After equilibration the reaction was started by the addition of 6·7 mM-sodium α-oxoglutarate, pH 7·5, bringing the total volume to 3 ml. *E* at 340 mμ was plotted against time in minutes and the result calculated as μmoles of DPNH oxidized/hr. at 25°.

### *Separation of enzyme fractions from rat liver*

*Subcellular fractionation.* Differential centrifuging of homogenates of rat liver in 0·25 M-sucrose was performed as described by Hogeboom (1955). Mitochondria were washed twice. Ultrasonic disintegration of subcellular fractions was carried out with a MSE–Mullard Ultrasonic Disintegrator, 60 w, with a frequency of approx. 20 keyc./sec., for 1 min. This instrument was tuned to give maximum agitation in the tissue preparation, which was kept immersed in ice-cold water.

*Electrophoretic fractionation.* The liver extract for electrophoresis was prepared by homogenizing rat liver in 10 vol. of ice-cold water in a Waring Blendor for 4 min. The insoluble material was removed by centrifuging for 30 min. at 20000*g* in a MSE refrigerator centrifuge. The supernatant was freeze-dried and redissolved in the electrophoresis buffer when required. In this extract approximately 60% of the original transaminase activity was recovered.

Paper electrophoresis was carried out on Whatman no. 541 paper on a Durram-type apparatus with 30 mM-$Na_2HPO_4$–$KH_2PO_4$ buffer, pH 7·5, *I* 0·1, 7 v/cm., 2 mA for 15 hr. at 4°.

Zone electrophoresis in a sucrose-density gradient was performed with a column electrophoresis apparatus, type LKB 3340, manufactured by LKB-Produkter, Fabriksaktiebolag, Stockholm, Sweden. 5 mM-$Na_2HPO_4$–$KH_2PO_4$ buffer, pH 7·5, *I* 0·03, was used with 650 v, 9 mA for 14 hr. at 15°.

### *Materials*

Purified malic dehydrogenase, α-oxoglutaric acid and DPNH were obtained from C. F. Boehringer und Soehne, Mannheim, W. Germany. L-Aspartic acid was obtained from L. Light and Co. Ltd., L-glutamic acid and D-aspartic acid from Roche Products Ltd. and oxaloacetic acid from Sigma Chemical Co., St Louis, Mo., U.S.A. All these substances were used without further purification.

## RESULTS

*Latency of L-glutamate–oxaloacetate transaminase in rat-liver homogenates.* Table 1 shows the effect of various treatments on the activity of transaminase in rat-liver homogenates. Parallel determinations were made of glutamic dehydrogenase. This was selected as a reference enzyme since it is established as a latent mitochondrial enzyme (Bendall & de Duve, 1960).

Hypo-osmoticity (homogenizing in water), mechanical disintegration of subcellular particles (5 min. in Waring Blendor) and ultrasonic disintegration (1 min.) produced a great increase in activity as measured by the Karmen method. Incubation at 37° for several hours, storage at 4° for several days or treatment with the detergent Triton X-100, 0·06% (w/v), also caused this activation observed by the Karmen method.

The rates of activation of glutamic dehydrogenase and transaminase by mechanical disintegration, are shown in Fig. 1. The explanation of the

activation of glutamic dehydrogenase suggested by Bendall & de Duve (1960) is that the enzyme is largely inaccessible to its substrates until the mitochondrial membrane is damaged. They have shown that this enzyme is liberated into solution by blending but that the increased activity after exposure to media of low concentration is not in solution. Table 2 shows that transaminase is similarly rendered soluble by mechanical and ultrasonic disintegration and not by hypo-osmotic media.

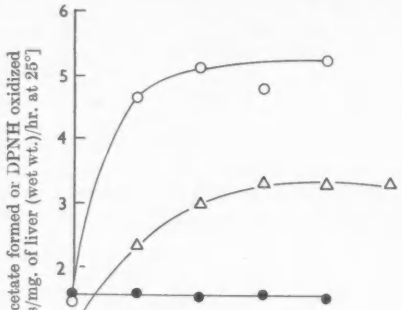


Fig. 1. Effect of mechanical disintegration in a Waring Blendor of a homogenate of rat liver in 0·25 M-sucrose on the glutamic-dehydrogenase and L-glutamic–oxaloacetate-transaminase activities. △, Glutamic dehydrogenase; ●, transaminase (method of Reitman & Frankel, 1957); ○, transaminase (method of Karmen, 1955).

The next step was to see if this latent transaminase activity was attached to certain subcellular particles as with glutamic dehydrogenase.

*Intracellular localization of* L-*glutamate–oxaloacetate transaminase.* Rat-liver homogenates were fractionated by differential centrifuging and the various cell fractions assayed for transaminase activity before and after ultrasonic disintegration. Once again glutamic dehydrogenase was used as a reference enzyme. Table 3 shows that the latent transaminase resides mainly in the mitochondrial fraction. With the Karmen method there is an increase of 120 % in activity in mitochondria after disintegration.

*Electrophoretic mobility of* L-*glutamate–oxaloacetate transaminase on paper.* For these experiments samples (5 μl.) of liver extract equivalent to 5 mg. of liver (wet wt.) were used. After electrophoresis some strips of paper were stained for protein with 1 % Light Green and others were cut into sections 5 mm. wide and the protein was eluted with 0·15 M-sodium chloride. These eluates were then assayed for transaminase. Over 90 % of the activity could be recovered from the paper. The

Table 2. *Solubility of* L-*glutamate–oxaloacetate transaminase in liver homogenates after activation procedures*

Transaminase was assayed by the method of Karmen (1955). Units are μmoles of oxaloacetate produced/hr./mg. of liver at 25°. For experimental details see text.

| Treatment | Activity before centrifuging | Centrifuged for 30 min., 20000*g* | | Recovery (%) |
|---|---|---|---|---|
| | | Deposit | Supernatant | |
| Water homogenate | 4·20 | 3·10 | 0·81 | 93 |
| Sucrose homogenate | | | | |
| Untreated | 2·04 | 2·19 | 0·73 | 142 |
| Waring Blendor, 4 min. | 5·63 | 2·00 | 2·84 | 86 |
| Ultrasonic disintegrator, 1 min. | 6·15 | 1·58 | 3·92 | 90 |

Table 3. *Distribution of glutamic dehydrogenase and* L-*glutamate–oxaloacetate transaminase in subcellular fractions of rat-liver homogenates, showing the effect of subsequent ultrasonic disintegration*

Units are μmoles of DPNH oxidized or oxaloacetate formed/hr./mg. of liver (wet wt.) at 25°. Transaminase was assayed by the method of Karmen (1955) and glutamic dehydrogenase as described in the text.

| Enzyme | Glutamic dehydrogenase | | L-Glutamate–oxaloacetate transaminase | |
|---|---|---|---|---|
| Ultrasonic disintegration (min.) ... | — | 1 | — | 1 |
| Whole homogenate | 0·51 | 1·66 | 3·10 | 6·24 |
| Nuclear fraction | 0·06 | 0·17 | 0·27 | 0·71 |
| Mitochondrial fraction | 0·56 | 1·56 | 1·45 | 3·25 |
| Microsomal fraction | 0·06 | 0·02 | 0·19 | 0·30 |
| Supernatant fraction | 0·13 | 0·04 | 1·31 | 1·23 |
| Recovery in fraction (%) | 159 | 108 | 104 | 88 |

results (Fig. 2) confirm the findings of Fleisher *et al.* (1960) that L-glutamate–oxaloacetate-transaminase activity separates into two peaks. One peak (fraction A) was not far from the point of application; the other peak (fraction B) was further from this point; both had moved towards the anode. Fraction A was in a similar position to $\gamma$-globulin, and fraction B to $\alpha$-globulin, of bovine serum on a parallel strip. This suggests that if allowance were made for endosmosis then fraction A would be shown to have moved towards the cathode. It is demonstrated in Fig. 3 that the transaminase of mitochondrial extract after electrophoresis is almost all in one peak, that corresponding to fraction A. The transaminase of supernatant fraction forms a major peak corresponding to fraction B. Its minor fraction A peak could be due to contamination with transaminase from mitochondria during homogenization.

*Substrate affinities and* pH*-dependence of* L*-glutamate–oxaloacetate transaminase.* Fleisher *et al.* (1960) showed that the two transaminases which they had separated from canine heart had different substrate affinities and pH-dependence curves. Table 4 shows that by the Karmen (1955) method the substrate affinities of two electrophoretic transaminases from rat liver also differ markedly. Moreover, the Michaelis constants of mitochondrial transaminase correspond fairly closely with those of fraction A. The supernatant transaminase and fraction B also correspond in this respect. Fig. 4 shows that with the Karmen method mitochondrial transaminase and fraction A are similar to one another and differed from supernatant transaminase and fraction B in their pH-dependence curves. Both the supernatant transaminase and fraction B have only 40% of maximum activity at pH 6·0. These results demonstrate the presence in the cell of two L-glutamate–oxaloacetate transaminases with different properties, one located principally in the mitochondria and the other in the supernatant fraction.

*Comparison of methods of* L*-glutamate–oxaloacetate-transaminase assay.* There is a large discrepancy between the results obtained by the method of Reitman & Frankel (1957) and those by the method of Karmen (1955) (Figs. 1, 2). The method of Reitman & Frankel gave very low results for fraction A. Table 5 shows that this method gave a similarly low estimate of the transaminase activity of mitochondria.

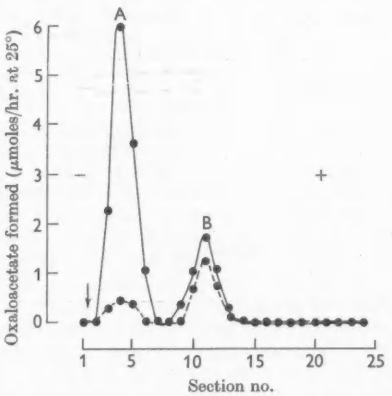


Fig. 2. Distribution of L-glutamate–oxaloacetate transaminase after paper electrophoresis of crude liver extract. A preparation containing 18·9 units (Karmen, 1955, method) and 8·9 units (Reitman & Frankel, 1957, method) of transaminase was applied near the cathode as indicated by the arrow, and recoveries after electrophoresis were 92 and 99% respectively. ●—●, Transaminase by method of Karmen (1955); ●- - -●, transaminase by method of Reitman & Frankel (1957).

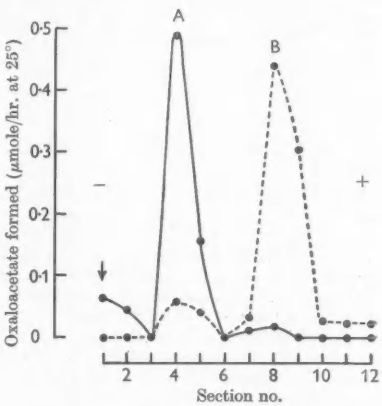


Fig. 3. Distribution of L-glutamate–oxaloacetate transaminase after paper electrophoresis of supernatant fraction (●- - -●) and mitochondrial extract (●—●) from rat liver. The two curves have been superimposed on one another so that their points of application (shown by an arrow) coincide. The supernatant fraction was obtained by centrifuging a 10% sucrose homogenate at 20000*g* for 30 min. The mitochondrial extract was prepared by subjecting the resuspended pellet, after washing it twice, to ultrasonic disintegration (1 min.) and removing the insoluble material by centrifuging at 20000*g* for 30 min. These preparations were applied in 5 $\mu$l. samples. Purified bovine serum albumin (1%) was added to the buffer used in order to stabilize the enzyme. The sections of paper were 10 mm. wide and were added directly to the assay mixture because of the low concentration of enzyme. Otherwise the experimental details were as described in the text.

Table 4. *Michaelis constants for* L-*glutamate–oxaloacetate transaminase in fractions from rat liver*

The Karmen method was used and $K_m$ values obtained from Lineweaver–Burk plots. All points fell satisfactorily near a straight line. Fractions A and B were obtained by paper electrophoresis of rat-liver extract.

| Transaminase fraction | No. of units of enzyme used (μmoles of oxaloacetate/hr. at 25°) | $K_m$ for α-oxoglutarate (mM with 33·3 mM-L-aspartate) | $K_m$ for L-aspartate (mM with 6·66 mM-α-oxoglutarate) |
|---|---|---|---|
| Mitochondrial* | 1·4 | 1·00 | 0·47 |
| Fraction A | 1·3 | 0·95 | 0·36 |
| Supernatant | 0·6 | 0·20 | 2·15 |
| Fraction B | 0·5 | 0·16 | 3·70 |

* Activated by ultrasonic disintegration for 1 min.

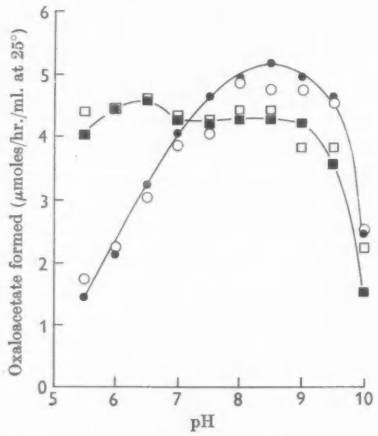


Fig. 4. Effect of change of pH on activity of L-glutamate–oxaloacetate transaminase as assayed by the method of Karmen (1955). The pH was altered from 7·5 by the addition of small amounts of N-HCl or N-NaOH to the substrates. □, Mitochondrial fraction (activated by ultrasonic disintegration); ○, supernatant fraction; ■, fraction A; ●, fraction B. Fractions A and B were obtained by density-gradient electrophoresis.

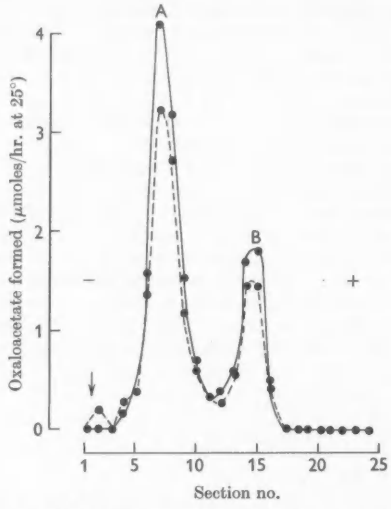


Fig. 5. Distribution of L-glutamate–oxaloacetate transaminase after paper electrophoresis of crude liver extract. A preparation containing 16 units by the method of Karmen (1955) was applied near the cathode as indicated by the arrow; 104% of this activity was recovered. ●—●, Transaminase (method of Karmen, 1955); ●- - -●, transaminase (method of Cammarata & Cohen, 1951, modified as described in the text).

In an attempt to resolve the difference between the results of the method of Reitman & Frankel and the method of Karmen, the subcellular and electrophoretic fractions were assayed by the method of Cammarata & Cohen (1951). The results in Fig. 5 and Table 5 show a close resemblance to the results of the method of Karmen. In the method of Cammarata & Cohen substrate concentrations were increased because with 20 mM-α-oxoglutarate only 70% of the total activity of fraction A was measured and 20 mM-L-aspartate was suboptimum for fraction B, giving only 80% of total activity. The α-oxoglutarate concentration needed was much higher than in the Karmen method, indicating that fraction A had a much lower affinity for α-oxoglutarate in the assay procedure of Cammarata & Cohen than in the Karmen method. The same was true, but to a much lesser extent, with fraction B. On the other hand, affinity for L-aspartate was not significantly affected by the assay procedure.

The progress curves with the Cammarata & Cohen method were linear for fraction B and supernatant transaminases but non-linear for fraction A and mitochondrial transaminase (Fig. 6). The progress curves for all transaminase fractions were linear in the Karmen method in which oxaloacetate

Table 5. *Distribution of* L-*glutamate–oxaloacetate transaminase in subcellular fractions of rat-liver homogenates assayed by different methods and the effect of ultrasonic disintegration*

Units are μmoles of oxaloacetate or glutamate formed/hr./mg. of liver (wet wt.) at 25°.

| Assay method ... ... | Cammarata & Cohen (1951) | | Reitman & Frankel (1957) | | Müller & Leuthardt (1950) | |
|---|---|---|---|---|---|---|
| Ultrasonic disintegration (min.) ... | — | 1 | — | 1 | — | 1 |
| Whole homogenate | 2·32 | 4·74 | 1·28 | 1·58 | 1·05 | 1·12 |
| Nuclear fraction | 0·34 | 0·94 | 0·05 | 0·05 | — | — |
| Mitochondrial fraction | 0·56 | 3·44 | 0·29 | 0·38 | 0·25 | 0·57 |
| Microsomal fraction | 0·40 | 0·38 | 0·01 | 0·02 | — | — |
| Supernatant fraction | 0·80 | 0·76 | 1·11 | 1·10 | 0·67 | — |
| Recovery in fractions (%) | 91 | 116 | 95 | 98 | — | — |

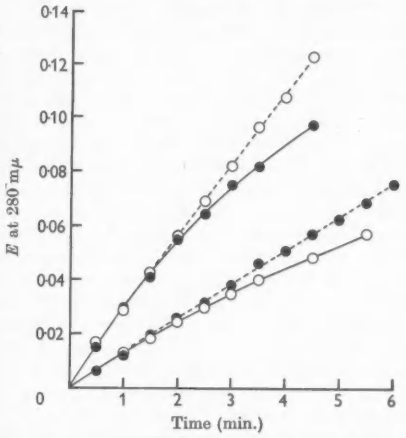


Fig. 6. Progress curves for L-glutamate–oxaloacetate-transaminase fractions assayed by a modification of the method of Cammarata & Cohen (1951). ●—●, Mitochondrial transaminase (activated by ultrasonic disintegration); ○—○, fraction A; ○- - -○, supernatant transaminase; ●- - -●, fraction B.

formed is removed. These results suggest that when oxaloacetate accumulates it inhibits transamination by fraction A and mitochondrial transaminase. The finding that affinities for α-oxoglutarate are lower in the Cammarata & Cohen method, particularly with mitochondria and fraction A, indicates that the oxaloacetate inhibition may be partially competitive.

It is concluded that the failure of the method of Reitman & Frankel can be attributed to the low α-oxoglutarate concentration (2 mM) and to inhibition by oxaloacetate.

*L-Glutamate formation by* L-*glutamate–oxaloacetate-transaminase fractions.* In order to prove that oxaloacetate measured in the above-described methods was formed by a transamination reaction, it was necessary to show that L-glutamate was being

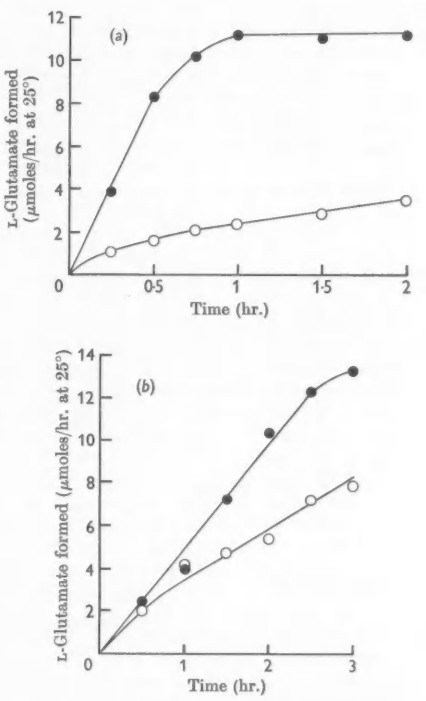


Fig. 7. (*a*) Effect of oxaloacetate reduction on activity of fraction A. A sample of fraction A capable of producing 14 μmoles of oxaloacetate/hr., as measured by the method of Karmen (1955), was assayed for L-glutamate production by means of glutamic decarboxylase (○). Acceleration was achieved by the addition of 5 μg. of purified malic dehydrogenase and 10 μmoles of DPNH (●). (*b*) A similar experiment with fraction B capable of producing 4·6 μmoles of oxaloacetate/hr. as measured by the method of Karmen (1955). The fractions used in this experiment were obtained by density-gradient electrophoresis, details of which are given in the text.

formed at the same rate. For this purpose the method of Müller & Leuthardt (1950) was examined. The concentrations of substrates were found to be suboptimum and were increased. Nevertheless, the glutamate formed in this method was much less than the oxaloacetate formed in the methods of Karmen and Cammarata & Cohen with both mitochondria and supernatant fractions (Table 5) and with electrophoretic fractions (Fig. 7). However, owing to the relative insensitivity of this assay procedure, much higher concentrations of oxaloacetate were accumulating than in the method of Cammarata & Cohen. When purified malic dehydrogenase and DPNH were added to the reaction mixture to reduce the oxaloacetate during the reaction, as in the Karmen method, glutamate production by fraction A and fraction B reached the concentrations expected and had a linear progress curve as long as DPNH was available (Fig. 7). The rate of L-glutamate production over the linear part of the curve for fraction A was 16·5 $\mu$moles/hr. compared with 14 $\mu$moles of oxaloacetate/hr., whereas the respective rates for fraction B were 4·9 and 4·6 $\mu$moles/hr.

## DISCUSSION

Hird & Rowsell (1950) pointed out that preoccupation with soluble transaminase preparations had tended to divert attention from the more general transamination reactions of the cell. They found that the glutamate–phenylpyruvate transaminase was located solely in the insoluble-particle fraction of rat-liver homogenates, and that transamination to form tyrosine, alanine and aspartate was also catalysed by these particle suspensions. The experiments reported here show that previous work on the distribution of L-glutamate–oxaloacetate transaminase in the cell has led to the under-estimation of the importance of transamination between L-aspartate and α-oxoglutarate in mitochondria. The main reason for this underestimation appears to be the latency of this transaminase in undamaged mitochondria. A contributory cause has been the failure of certain assay methods to measure the true activity of mitochondrial L-glutamate–oxaloacetate transaminase. This failure is shown to be due in part to the fact that mitochondrial transaminase differs in substrate affinities and other properties from supernatant transaminase. In three of four commonly used assay procedures substrate concentrations were found to be suboptimum, particularly for the mitochondrial enzyme. Furthermore, transamination from aspartate is inhibited as oxaloacetate produced accumulates. This inhibition, which is apparently partly due to oxaloacetate competing with α-oxoglutarate for the enzyme, affects the mitochondrial enzyme more than the supernatant one. Only the Karmen (1955) method, in which oxaloacetate formed is reduced to malate by added malic dehydrogenase and DPNH, gives linear progress curves at the optimum rate with mitochondrial transaminase. The results from the Karmen (1955) and modified Cammarata & Cohen (1951) methods show that there is three or four times as much L-glutamate–oxaloacetate transaminase in the mitochondria after activation as in the supernatant fraction of liver cells.

A large number of investigators have studied changes in L-glutamate–oxaloacetate-transaminase activity in the tissues and body fluids of patients with various diseases and of experimental animals subjected to various interferences, e.g. treatment with hormones. Unfortunately, as well as using the unsatisfactory assay procedures discussed above, many workers in this field have used fresh isosmotic tissue preparations kept refrigerated and stored for the minimum time. Hence changes in activity due to changes confined to the mitochondrial enzyme may have been underestimated, whereas elevations in activity recorded may have been merely due to unmasking of latent enzyme.

The discovery that mitochondrial transaminase differs from supernatant transaminase in substrate affinities, pH-dependence and electrophoretic mobility allows an interesting parallel to be drawn between L-glutamate–oxaloacetate transaminase and malic dehydrogenase, which has recently been shown to exist in two forms, one localized in the mitochondria and the other in the supernatant fraction of rat-liver homogenates (Thorne, 1960). Further work is necessary to ascertain whether the two types of enzyme are in fact distinctly different proteins. Sophianopaulus & Vestling (1960) have claimed that the supernatant form of malic dehydrogenase may be converted into the mitochondrial form simply by treatment with butan-1-ol.

It is possible that further purification might reveal the existence of other types of L-glutamate–oxaloacetate transaminase. In preliminary experiments it has been found that whereas fraction B and supernatant fractions of transaminase can react only with L-aspartate, both fraction A and mitochondrial fractions react also with D-aspartate although at a slower rate and with lower affinity. It has not yet been found whether this results from lack of stereospecificity in the mitochondrial enzyme or whether there is a further enzyme which reacts with the D-amino acid.

## SUMMARY

1. Methods of L-glutamate–oxaloacetate-transaminase assay (with α-oxoglutarate and L-aspartate as substrates) have been compared and modified. Maximum activity could be readily achieved only

with the assay system of the method of Karmen (1955).

2. L-Glutamate–oxaloacetate transaminase has been shown to be present in rat-liver mitochondria in latent form. After activation the activity of transaminase in mitochondria is three to four times that in the supernatant fraction.

3. Activation has been achieved by using hypo-osmotic media, incubation at 37°, storage at 4°, treatment with Triton X-100, mechanical disintegration and ultrasonic disintegration. With ultrasonic and mechanical disintegration, but not with hypo-osmotic treatment, this activation is accompanied by release of the enzyme in soluble form.

4. It has been shown that mitochondrial transaminase differs from supernatant transaminase in its substrate affinities, pH-dependence and electrophoretic mobility.

5. Two L-glutamate–oxaloacetate transaminases separated electrophoretically from rat-liver extract appear to correspond to the mitochondrial and supernatant enzymes respectively.

6. Transamination from L-aspartate is inhibited by the oxaloacetate produced.

I wish to acknowledge with thanks the helpful advice and criticism received from Dr G. D. Greville, and the technical assistance given by Miss A. Saltmarsh and Mr R. Braham. My thanks are also due to Dr A. D. Bangham and Mr R. J. Hall for advice and assistance with the electrophoretic procedures.

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# Characterization of Tissue Alkaline Phosphatases and their Partial Purification by Starch-Gel Electrophoresis

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Attempts have been made to assign the alkaline phosphatase present in abnormal amounts in human blood plasma in certain diseases to an origin in a given tissue by a study of the properties of the enzyme increment. These attempts have yielded only inconclusive results. [References may be found in the recent review by Gutman (1959).] Studies of this nature are impeded by the presence in serum of inhibitors such as albumin (Henneman, Rourke & Jackson, 1955) and bile acids (Bodansky, 1937), of activators, such as metal cations, and of a factor of an unknown nature which appears to be present in icteric serum (Cantarow, 1940). Amino acids may inhibit or activate depending on their concentration (Bodansky, 1946). The properties of alkaline phosphatase in serum may reflect therefore not so much the properties of the enzyme as those of its environment.

Electrophoresis on starch gel (Smithies, 1955) permits a refined separation of the serum proteins

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to be made, although the amounts of protein separated are small. The resolving power of electrophoretograms run in the conventional manner on horizontal gels declines if the sample volume exceeds about 0·1 ml. of serum/cm.$^2$ of gel cross-section, and this limits the use of the method for preparative purposes. However, this paper shows that if a sufficiently sensitive technique for measuring enzyme activity is available, reproducible values for the Michaelis constant of alkaline phosphatase purified from serum by starch-gel electrophoresis can be obtained. By these means it has been possible to isolate in a semi-pure state the alkaline phosphatases from tissue preparations and from blood plasma and to study their properties.

## EXPERIMENTAL

*Enzyme preparations.* Recent post-mortem human tissues, bone, liver, kidney and small intestine were used as sources of alkaline phosphatase. After cleaning and grinding, the tissues were kept in 5 vol. of chloroform–water. When activity had reached a maximum (2–5 days) each solution was cleared of solid matter by centrifuging, and the supernatant was poured into 5 vol. of ethanol–ether (3 : 2, v/v). The precipitate was collected by centrifuging and was immediately redissolved in normal human blood serum or in 0·9% NaCl solution. The precipitate from bone was further purified by grinding in a mortar with a small volume of 50% ethanol. The filtered or centrifuged 50% ethanol solution was treated with ethanol–ether to give a final composition of 3 vol. of ethanol, 2 vol. of ether and 1 vol. of water. The precipitate that formed was recovered by centrifuging and was similarly redissolved, either in normal serum or in 0·9% NaCl solution.

Serum from a case of Paget's disease with alkaline-phosphatase activity 65 King–Armstrong (1934) units/100 ml. was also used as a source of bone alkaline phosphatase.

*Starch-gel electrophoresis.* This was carried out in horizontal trays measuring 17 cm. × 10·5 cm. with a gel thickness of about 0·4 cm. essentially by the method of Smithies (1955). The gels were prepared in tris–citrate buffer (Poulik, 1957), except when specific activities of enzymes recovered from the gels were determined, when the gels were made in dilute $H_3BO_3$–NaOH buffer (Smithies, 1955). In each case, concentrated $H_3BO_3$–NaOH buffer (Smithies, 1955) was used in the electrode chambers. The sample was applied on a filter-paper support; when nearly the full width of the gel was used, it was possible to run samples of about 0·2 ml. After electrophoresis at 20 v/cm. for 5–6 hr. the alkaline-phosphatase band was located in the following way: strips of filter paper, moistened with a solution of disodium α-naphthyl phosphate (5 mM) in $Na_2CO_3$–$NaHCO_3$ buffer, pH 10 (Delory & King, 1945), containing 5 mM-$MgCl_2$, were laid along the longitudinal edges of the two cut surfaces of the gel so that the papers just overlapped the edges of the electrophoresis zones. After incubation at 37° (usually 5–10 min. was sufficient) the papers were removed and the gels were viewed under an ultraviolet lamp. The ends of the alkaline-phosphatase zones were then visible as areas of pale-blue fluorescence in the dark-blue fluorescent margins. The narrow strip across the gel between the two ends of the phosphatase zone was removed for elution of the enzyme. The ends of the zones where α-naphthyl phosphate hydrolysis had taken place were avoided in cutting because of their contamination with α-naphthol and its phosphate and consequent marked fluorescence. α-Naphthyl phosphate was preferred to the β-isomer for the visual location of phosphatase, since the fluorescence of α-naphthol (emission max. 455 mμ) is more apparent to the eye than is that of β-naphthol.

Phosphatase was recovered from the gel by macerating the enzyme-containing strips with $Na_2CO_3$–$NaHCO_3$ buffer, pH 10, and then centrifuging (for the two strips of gel obtained from one electrophoresis, 2 ml. of buffer was used and yielded 0·7–1 ml. of supernatant). The enzyme solution was used immediately. This method of recovering the enzyme gave approximately the same activity in the volume of solution needed for a Michaelis-constant determination (1·25 ml.) as did freezing, thawing and centrifuging the gel, which necessitated greater subsequent dilution of the enzyme solution expressed. Overall enzyme recoveries of up to 30% were obtained, allowance being made for the volume of buffer retained by the gel after centrifuging.

For specific-activity determinations, nitrogen was estimated in the phosphatase solution recovered from gels made in $H_3BO_3$–NaOH buffer by Kjeldahl digestion followed by nesslerization (a piece of gel from a protein-free region of the same weight as the phosphatase strip was treated with buffer to provide a control). Phosphatase activity was estimated in the same solution by the Kind & King (1954) modification of the King & Armstrong (1934) method to enable comparison to be made with other purified enzyme preparations.

*Determination of Michaelis constant.* Disodium β-naphthyl phosphate was used as substrate. This was prepared by the method of Friedman & Seligman (1950), and was recrystallized (Found: C, 43·8; H, 3·2; P, 11·0; Na, 17·4. Calc. for $C_{10}H_7O_4PNa_2$: C, 44·8; H, 2·6; P, 11·6; Na, 17·1%). This ester is hydrolysed by serum alkaline phosphatase at a rate approximately 0·75 that of phenyl phosphate, with liberation of free β-naphthol. The fluorescence excitation and emission spectra (Fig. 1) of β-naphthol and of its orthophosphate ester differ sufficiently in alkaline solution to permit the measurement of traces of β-naphthol in the presence of a large excess of the ester, by using a spectrofluorimeter with activation wavelength set at 350 mμ and emission measurement at 420 mμ (Moss, 1960*a*).

The spectrofluorimeter used in the present experiments employs quartz-prism monochromators, xenon-arc light-source and photomultiplier with pulse-counting circuits as detector. The output of the sample is measured in terms of the output of a fluorescent screen which monitors the activating beam (Moss, 1960*b*).

For measurement of β-naphthol an activation band width of 5 mμ was used, with emission band width of either 5 or 15 mμ. Calibration curves at the two settings in terms of μmoles of β-naphthol in a volume of 3 ml. are given in Fig. 2. With the 15 mμ emission band width the mean value of nine determinations of 0·2 μm-mole of β-naphthol, expressed as a percentage, was 102 ± 3% (S.D.).

All enzymic reactions were carried out at 37°. The range of substrate concentration used was 0·05–1 mM, and the

reaction mixtures were buffered with $Na_2CO_3$–$NaHCO_3$ buffers (Delory & King, 1945), except in a few cases (indicated in the results) in which the $Na_2CO_3$–veronal buffers (King & Delory, 1940) were used.

The solutions were made 5 mM with respect to $Mg^{2+}$ ions by addition of an appropriate volume of 0·1 M-$MgCl_2$. Before addition of enzyme the volume of the buffered reaction mixture was 2 ml.; the reaction was started by addition of 0·05 ml. of enzyme solution from a constriction pipette. With the crude tissue extracts the reaction was stopped after 5 min. by addition of 1 ml. of a mixture of 8 vol. of 0·5 N-NaOH and 2 vol. 0·1 M-EDTA; for the enzyme recovered from the gels hydrolysis was allowed to proceed for 15 min. Addition of NaOH brings the pH to about 12, at which alkaline phosphatase is inactive and β-naphthol is strongly fluorescent (the fluorescence of β-naphthol measured at the chosen wavelengths reaches a maximum, constant value at pH 10 and above). The EDTA keeps $Mg^{2+}$ ions in solution; in the absence of EDTA a gelatinous precipitate of $Mg(OH)_2$ forms. Controls were set up in which buffered substrate at each concentration used was incubated for the same time as the tests, enzyme solution being added at the end of incubation immediately after addition of the NaOH–EDTA. The blank fluorescence depends almost entirely on substrate concentration, being due partly to traces of free β-naphthol present in the phosphate, but also to the inherent fluorescence of the phosphate itself. Non-enzymic hydrolysis during the 5 or 15 min. incubation was in fact negligible. A pH-optimum curve was determined for each substrate concentration with each enzyme preparation, and the optimum velocities at each concentration were used in constructing the Lineweaver–Burk (1934) reciprocal plot (Motzok, 1959). Straight lines were fitted to the experimental points by the method of least squares. The term $K_m$ is used in this paper to denote the parameters derived from the intercepts of these lines on the abscissae.

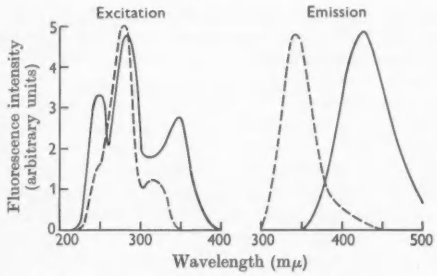


Fig. 1. Fluorescence excitation and emission spectra of β-naphthol (——) and β-naphthyl phosphate (- - -), at pH 10. The excitation spectra have been corrected to constant quantum intensity. Excitation maxima: β-naphthol, 250, 285 and 350 mμ; β-naphthyl phosphate, 280 and 320 mμ. Emission maxima: β-naphthol 425 mμ; β-naphthyl phosphate, 340 mμ.

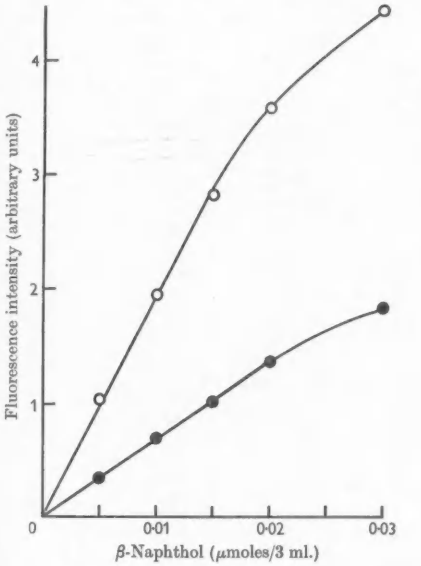


Fig. 2. Relation between concentration of β-naphthol and fluorescence, at pH 12. Fluorescence excitation, 350 mμ; emission measured at 420 mμ. Excitation band width, 5 mμ; emission band width: ●, 5 mμ; ○, 15 mμ.

For the enzyme solutions recovered from the gels only the points on each pH–activity curve needed to define the optima were determined, since the volume of solution available was limited.

*Inhibition by sodium deoxycholate.* The effect of sodium deoxycholate on the hydrolysis of β-naphthyl phosphate by alkaline phosphatase extracts from bone, liver and intestine was investigated as a possible additional means of differentiating between the enzymes from different tissues. A 1 mM-substrate concentration and 5 mM-deoxycholate were used, $Mg^{2+}$ ions being 5 mM and pH 9·7 at 37°. The velocity of hydrolysis in the presence of deoxycholate was compared with that in controls from which deoxycholate was omitted.

## RESULTS

The major portion of human serum alkaline phosphatase has a mobility on starch-gel electrophoresis which is slightly less than that of the transferrin-C (β-globulin) protein fraction (Kowlessar, Pert, Haeffner & Sleisenger, 1959; Estborn, 1959). The tissue phosphatases prepared in the present study gave a main band of activity in this region of the gel, whether dissolved in serum or 0·9% sodium chloride solution, and the Michaelis constants reported here of electrophoretically purified enzymes refer to phosphatases recovered from this zone of the gels. A phosphatase fraction, moving rather more slowly than the slow $\alpha_2$-protein fraction, that was present in certain preparations, notably from intestine, has not yet been investigated fully.

Table 1 shows that partial purification by starch-gel electrophoresis considerably changed the Michaelis constants of the liver and kidney enzymes. The values for the bone and intestinal enzymes were less affected. The $K_m$ values were in good agreement whether the tissue phosphatase was submitted to electrophoresis as a solution in serum or in 0·9% sodium chloride solution. Bone

Table 1. *Michaelis constants for hydrolysis of β-naphthyl phosphate by tissue alkaline phosphatases, before and after partial purification by starch-gel electrophoresis*

Temp. 37°; $Mg^{2+}$, 5 mM. Velocity at optimum pH for each substrate concn. used in determining $K_m$. c, $Na_2CO_3$–$NaHCO_3$ buffers; v, veronal-$Na_2CO_3$ buffers; sa, submitted to electrophoresis in solution in 0·9% NaCl; se, submitted to electrophoresis in solution in normal human serum; p, Paget's-disease serum.

| | Before electrophoresis | | | After starch-gel electrophoresis | | |
|---|---|---|---|---|---|---|
| Tissue of origin of phosphatase | $K_m$ (mM) | $V_{max}$ for enzyme solution used in $K_m$ determination (μmoles/min./l.) | Mean $K_m$ (mM) | $K_m$ (mM) | $V_{max.}$ for enzyme solution used in $K_m$ determination (μmoles/min./l.) | Mean $K_m$ (mM) |
| Bone | 0·101 (v) | 48 | 0·103 | 0·110 (c, sa) | 3·5 | 0·110 |
| | 0·091 (v) | 98 | | 0·110 (c, se) | 1·2 | |
| | 0·116 (c) | 84 | | 0·110 (c, p) | 9·0 | |
| Liver | 0·149 (v) | 40 | 0·139 | 0·075 (c, sa) | 2·7 | 0·067 |
| | 0·137 (v) | 30 | | 0·071 (c, se) | 18 | |
| | 0·132 (c) | 82 | | 0·063 (c, se) | 17 | |
| | | | | 0·060 (c, se) | 10 | |
| Intestine | 0·097 (c) | 96 | 0·099 | 0·088 (c, sa) | 26 | 0·090 |
| | 0·100 (c) | 112 | | 0·095 (c, sa) | 13 | |
| | | | | 0·087 (c, se) | 2·9 | |
| Kidney | 0·133 (c) | 98 | 0·167 | 0·100 (c, sa) | 6·1 | 0·103 |
| | 0·200 (c) | 125 | | 0·105 (c, se) | 3·3 | |

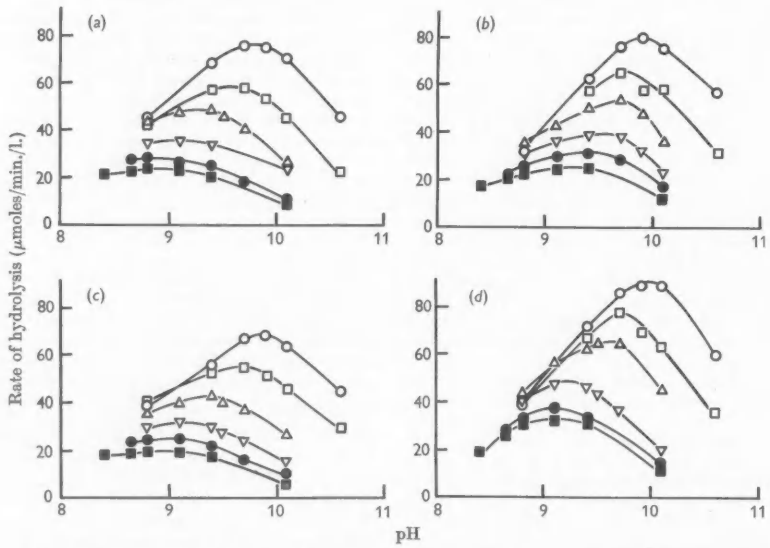


Fig. 3. pH–Activity curves for alkaline phosphatases of tissue extracts at various substrate concentrations. β-Naphthyl phosphate concn.: ○, 1 mM; □, 0·4 mM; △, 0·2 mM; ▽, 0·1 mM; ●, 0·067 mM; ■, 0·05 mM. (*a*), Bone; (*b*), kidney; (*c*), liver; (*d*), intestinal phosphatase. Temp. 37°; $Mg^{2+}$, 5 mM.

alkaline phosphatase recovered from Paget's-disease serum gave a $K_m$ identical with that of the tissue enzyme added to normal serum and recovered by electrophoresis. The possibility of obtaining reproducible $K_m$ values on enzymes recovered from serum by starch-gel electrophoresis as an aid to identification of their tissues of origin was an important reason for undertaking this work.

Examples of pH–activity curves for the saline solutions of tissue alkaline phosphatases before electrophoresis are given in Fig. 3 and reciprocal plots derived from these data in Fig. 4. The pH–activity curves for enzymes purified by starch-gel electrophoresis and the corresponding reciprocal plots are shown in Figs. 5 and 6.

The $K_m$ values obtained were independent of the concentration of enzyme used, as is shown by the results for bone enzyme recovered from serum, the same $K_m$ being given by two preparations, one of which had a value of $V_{max.}$ approximately eight times that of the other. Similarly, good agreement in $K_m$ was obtained when liver and intestinal preparations having $V_{max.}$ values differing by a factor of 2 were used. Examples of the increase in specific activity obtained by starch-gel electrophoresis of the enzyme preparations, both in solution in normal serum and in 0·9% sodium chloride solution, are given in Table 2. The nitrogen content of the eluates from the gel was very low, and the specific activities in Table 2 are regarded as approximations only.

Sodium deoxycholate inhibited bone and liver phosphatase to the extent of 20–40% under the conditions used. Intestinal phosphatase was not inhibited, confirming the observation of Bodansky (1937).

## DISCUSSION

Considerable variation in the $K_m$ values of alkaline phosphatases from different tissues towards a given substrate has been reported, e.g. by Roche & Sarles (1948). These $K_m$ values were based on the change of velocity with substrate concentration at a constant pH. Motzok (1959) showed that if velocities at the optimum pH for each substrate concentration were used in deriving $K_m$ the data were resolved into two parts, giving a $K_m$ at low substrate concentrations and a second $K_m$ at high substrate concentrations; further, these $K_m$ values were independent of enzyme concentration. Motzok & Branion (1959) reported $K_m$

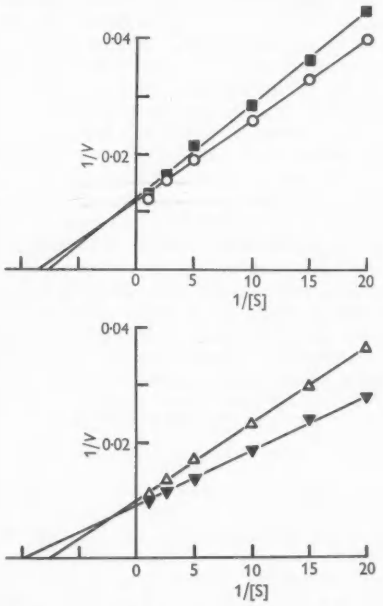


Fig. 4. Reciprocal plots ($1/V$, at optimum pH, against $1/[S]$) of data from Fig. 3. ○, Bone ($K_m$ 0·11 mM); ■, liver ($K_m$ 0·13 mM); △, kidney ($K_m$ 0·13 mM); ▼, intestinal phosphatase ($K_m$ 0·10 mM).

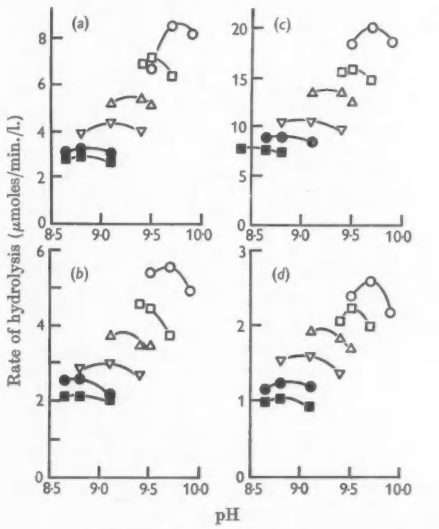


Fig. 5. pH–Activity curves for alkaline phosphatases partially purified by starch-gel electrophoresis. Substrate concentrations were as in Fig. 3. (*a*), Bone; (*b*), kidney; (*c*), liver; (*d*), intestinal phosphatase. Temp. 37°; $Mg^{2+}$, 5 mM.

values for several tissue alkaline phosphatases of rabbit and pigeon with sodium $\beta$-glycerophosphate as substrate determined both at constant pH and at optimum pH for each substrate concentration. Differences between the values of $K_m$ for different tissue phosphatases within the same species were less marked when optimum pH conditions for each substrate concentration were used than when a constant pH was maintained throughout. These authors conclude that, even if the concentrations of enzymes used in different determinations of $K_m$ at constant pH were kept the same, values so obtained would not be comparable because of the effect of differences in pH optima existing between phosphatases from different sources. Phosphatase $K_m$ values in the literature may only be applicable therefore to the particular enzyme preparations and concentrations employed in their determination. In the present investigation marked differences between the pH optima at a given substrate concentration were not found for phosphatases from the several human tissues used. However, it would be difficult to maintain reasonably constant enzyme concentration, on the basis of approximately equal activities, when studying solutions recovered from starch gels and tissue extracts of widely varying enzyme content, so determination of $K_m$ from velocity at optimum pH for each concentration of substrate was preferred since this has been shown to be little affected by enzyme concentration (Motzok, 1959). The values reported here would correspond to those found by Motzok for the region of dilute substrate.

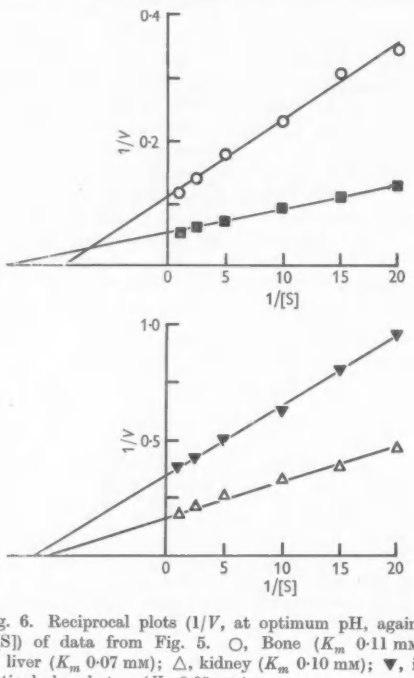


Fig. 6. Reciprocal plots ($1/V$, at optimum pH, against $1/[S]$) of data from Fig. 5. ○, Bone ($K_m$ 0·11 mM); ■, liver ($K_m$ 0·07 mM); △, kidney ($K_m$ 0·10 mM); ▼, intestinal phosphatase ($K_m$ 0·09 mM).

The $K_m$ values reported for alkaline phosphatase from different tissues acting on $\beta$-naphthyl phosphate show only small differences in the enzymes partially purified by starch-gel electrophoresis. The most marked difference is between the enzyme obtained from liver and those from other sources. Purification by electrophoresis produced, in liver and kidney phosphatases, a marked fall in $K_m$. A similar fall in $K_m$ is seen when the value of 0·11 mM obtained for bone enzyme recovered from Paget's disease serum is compared with the value of 0·4 mM obtained for whole Paget's-disease serum by similar methods (Moss, 1960*a*); these changes in $K_m$ emphasize the need for at least partial purification of serum enzymes in studies of this nature, because of the uncontrolled effect of the activator and inhibitors previously mentioned. However, the fall in $K_m$ consequent on electrophoresis of the phosphatase fractions of tissues or sera cannot be ascribed without qualification to a removal of inhibitors. The possibilities that electrophoresis produces some change in the enzyme molecule itself, or that it selectively destroys some

Table 2. *Increase in specific activity of alkaline phosphatase resulting from starch-gel electrophoresis*

Intestinal and liver phosphatases were in solution in normal serum; bone phosphatase was in solution in 0·9% NaCl.

| | Before electrophoresis | | | After starch-gel electrophoresis | | |
|---|---|---|---|---|---|---|
| Source of phosphatase | Activity (King–Armstrong units/100 ml.) | Nitrogen (mg./100 ml.) | Specific activity (units/mg. of N) | Activity (King–Armstrong units/100 ml.) | Nitrogen (mg./100 ml.) | Specific activity (units/mg. of N) |
| Intestine | 1020 | 800 | 1·3 | 11·3 | 0·5 | 25 |
| Liver | 54 | 1330 | 0·04 | 1·0 | 0·2 | 5 |
| Bone | 180 | 51 | 3·5 | 2·0 | 0·2 | 10 |

isoenzyme fractions having different affinity constants, or separates minor isoenzyme fractions which are afterwards undetected, cannot at present be discounted.

Although the partially purified enzyme solutions obtained from the starch gels do not compare in specific activity with the very highly purified alkaline phosphatases (e.g. Ahmed & King, 1960; Morton, 1954), which had activities of 1750 and 1470 King–Armstrong units/mg. of N respectively, electrophoresis on starch gel offers a method of eliminating, in one simple operation, large quantities of plasma protein and of concentrating the enzyme in a protein-poor solution to an enzymic strength comparable with that obtained by extracting enzyme-rich tissues. The properties of these enzyme preparations can be studied with techniques of adequate sensitivity. An increase in sensitivity of one or two orders of magnitude compared with colorimetric or spectrophotometric procedures is obtained where fluorimetric or spectrofluorimetric techniques can be applied.

A knowledge of the organs of origin of enzymes circulating in blood is of biochemical interest and in pathological states may also be of diagnostic value. The problem of identifying the tissue sources of alkaline phosphatase by investigating the properties of the enzyme purified from serum by starch-gel electrophoresis is complicated by the similar mobilities of various tissue phosphatases on the gel. Thus the enzyme solution recovered from the transferrin-C–haptoglobin region of an electrophoretogram of a pathological serum may contain a mixture of enzymes derived from various organs. The $K_m$ value of this solution would give a curved reciprocal ($1/V$ against $1/[S]$) plot, tending to give at low substrate concentration the $K_m$ of the enzyme with the highest affinity for the substrate. It may be possible further to fractionate this mixture by studying the kinetics of activation or inhibition of its component enzymes. Although the observations of Bodansky (1937) that sodium deoxycholate inhibits bone but not intestinal phosphatase have been confirmed, liver enzyme is also inhibited; therefore the use of this particular inhibitor does not assist in the differentiation of the enzymes recovered from starch gel into those originating in bone and in liver, and this is the most interesting problem in the study of pathological sera. Application of the methods described in this paper to the identification of the tissues of origin of the serum alkaline phosphatase increments in disease is described by Moss, Campbell, Anagnostou-Kakaras & King (1961). $K_m$ values of 0·11 mM (2) and 0·095 mM were found in bone disease, 0·094 mM in a case of extrahepatic biliary obstruction, and a range of values from 0·061 to 0·083 in seven cases with liver involvement.

## SUMMARY

1. Alkaline phosphatases from human bone, liver, kidney and intestine have been partially purified by starch-gel electrophoresis. These enzymes have also been recovered from solution in blood serum by electrophoresis.

2. The $K_m$ values for the alkaline phosphatases have been compared before and after electrophoresis and after recovery of the enzymes from serum, by using a spectrofluorimetric enzyme assay with $\beta$-naphthyl phosphate as substrate.

3. Reproducible $K_m$ values can be obtained for the tissue enzymes submitted to electrophoresis, and, although the differences in substrate affinity between phosphatases from individual tissues are small, these differences can be demonstrated after electrophoresis. Mean $K_m$ values (mM-$\beta$-naphthyl phosphate) found after electrophoresis were: bone, 0·110; liver, 0·067; intestine, 0·090; kidney, 0·103.

4. By combining starch-gel electrophoresis with spectrofluorimetric determination of phosphatase activity, alkaline phosphatases in blood serum can be studied with reduced interference from activators and inhibitors present in the serum.

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